DESIGN, SYNTHESIS, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF mTOR INHIBITORS FOR ANTICANCER ACTIVITY

A dissertation submitted to
The Tamilnadu Dr. M.G.R. Medical University
Chennai

In partial fulfillment of the requirements

for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL CHEMISTRY

Submitted by
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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
COLLEGE OF PHARMACY
MADRAS MEDICAL COLLEGE
CHENNAI – 600 003

MAY 2012

CERTIFICATE

This is to certify that the dissertation entitled "**DESIGN, SYNTHESIS, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF mTOR INHIBITORS FOR ANTICANCER ACTIVITY"** submitted by **26108334** in partial fulfillment of the requirements for the award of the degree of **MASTER OF PHARMACY** in **PHARMACEUTICAL CHEMISTRY** by the Tamilnadu Dr.M.G.R. Medical Univeristy is a bonafide work done by her during the academic year 2011-2012 at the Department of Pharmaceutical Chemistry, College of Pharmacy, Madras Medical College, Chennai – 600 003.

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Chennai – 600 003.

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This is to certify that KAVITHA S, Post Graduate student, Department of Pharmaceutical Chemistry, College of Pharmacy, Madras Medical College, Chennai-03 had submitted her protocol (PartB Application) vide 1/243 CPCSEA for the dissertation programme to the Animal Ethical Committee, Madras Medical College, Chennai-03.

TITLE

"DESIGN, SYNTHESIS, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF mTOR INHIBITORS FOR ANTICANCER ACTIVITY"

The Animal Ethical Committee experts screened her proposal vide 1/243 CPCSEA and have given clearance in the meeting held on 21.01.2012 at Dean's Chamber in Madras Medical College.

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CONTENTS

DEDICATED

TO

MY FAMILY

&

FRIENDS

ESPECIALLY

TOMMY

INTRODUCTION

1. INTRODUCTION[1, 2, 3, 4]

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Cancers arise approximately in one among every three individuals. DNA mutations arise normally at a frequency of 1 in every 20 million per gene per cell division. The average number of cells formed in any individual during an average lifetime is 10^{16} (10 million cells being replaced every second!). Risk of cancers are increased by infectious agents including viruses [Hepatitis B virus (HBV1), Human Papillomavirus (HPV), Human Immunodeficiency Virus(HIV)-increase risk of Nasopharyngeal, Cervical carcinomas and Kaposi's Sarcoma] and bacteria such as Helicobacter pylori (Stomach cancers).

Initiation and progression of cancer is also due to exposure to cancer-causing agents (carcinogens, mutagens). These are present in the food and water, in the air, and in chemicals and sunlight that people are exposed to. Since epithelial cells cover the skin, line the respiratory and alimentary tracts, and metabolize ingested carcinogens, it is not surprising that over 90% of cancers originate from epithelia (carcinomas). In less than 10% of cases, a genetic predisposition increases the risk of cancer developing a lot earlier. (e.g. Certain childhood leukemia's, retinal cancers etc.)

Although cancer can occur in persons of every age, it is common among the aging population. 60% of new cancer cases and two thirds of cancer deaths occur in persons >65 years. The incidence of common cancers (e. g. breast, colorectal, prostate, lung) increases with age. The exponential rise in many cancers with age fits with an increased susceptibility to the late stages of carcinogenesis by environmental exposures. Lifetime exposure to estrogen may lead to breast or uterine cancer; exposure to testosterone leads to prostate

cancer. The decline in cellular immunity may also lead to certain types of cancer that are highly immunogenic (e.g. lymphomas, melanomas). Accumulation of DNA mutations have to be amplified to constitute a cancer, therefore the longer the life span, the higher the risk of developing cancer.

The six hallmarks of cancers are:

- Immortality: continuous cell division and limitless replication
- Produce ‘Go’ signals (growth factors from oncogenes)
- Override ‘Stop’ signals (anti-growth signals from tumour suppressor genes)
- Resistance to cell death (apoptosis)
- Angiogenesis: Induction of new blood vessel growth
- Metastasis: Spread to other sites

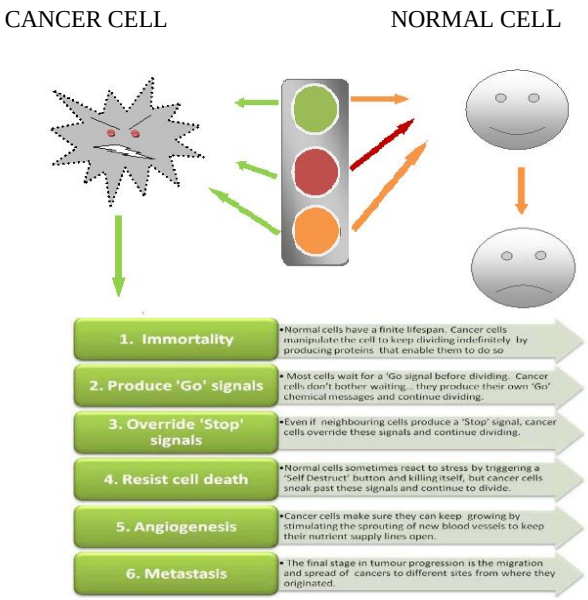


Fig.1 Hallmarks of cancer

Almost all cancers share some or all of the above mentioned six traits depending on the tumour.

The available anticancer drugs have distinct mechanisms of action which may vary in their effects on different types of normal and cancer cells. A single "cure" for cancer has proved elusive since there is not a single type of cancer but as many as 100 different types of cancer. In addition, there are very few demonstrable biochemical differences between cancerous cells and normal cells. For this reason the effectiveness of many anticancer drugs is limited by their toxicity to normal rapidly growing cells in the intestinal and bone marrow areas. A final problem is that cancerous cells which are initially suppressed by a specific drug may develop a resistance to that drug. For this reason cancer chemotherapy may consist of using several drugs in combination for varying lengths of time.

The majority of drugs used for the treatment of cancer today are cytotoxic (cell-killing) drugs that work by interfering in some way with the operation of the cell's DNA. Cytotoxic drugs have the potential to be very harmful to the body unless they are very specific to cancer cells - something difficult to achieve because the modifications that change a healthy cell into a cancerous one are very subtle. A major challenge is to design new drugs that will be more selective for cancer cells, and thus have lesser side effects. The reason for this is simple: cancer cells are not foreign to the body but are simply subtly mutated forms of normal human cells, and it is very different to synthesize drugs that can tell the difference.

Types of cancer:

Cancers may be categorized based on the functions/locations of the cells from which they originate. The following terms are commonly used to categorize by their tissue (cell type) of origin.

- ✓ Carcinoma- a tumor derived from epithelial cells, those cells that line the surface of our skin and organs. This is the most common cancer type and represents about 80-90% of all cancer cases reported.
- ✓ Sarcoma- a tumor derived from muscle, bone, cartilage or connective tissues.
- ✓ Leukemia- a cancer derived from white blood cells or their precursors. The cells that form both white and red blood cells are located in the bone marrow.
- ✓ Lymphoma- a cancer of bone marrow derived cells that affect the lymphatic system.
- ✓ Myelomas- a cancer involving the white blood cells responsible for the production of antibodies (B lymphocytes or B-cells)

Each type of cancer is unique with its own causes, symptoms, and method of treatment. The most common cancers are:

- ➢ Breast cancer
- ➢ Colorectal cancer
- ➢ Lung cancer
- ➢ Prostate cancer
- ➢ Skin cancer
- ➢ Bladder cancer
- ➢ Renal cell carcinoma

- Pancreatic cancer
- Leukemia

Globally, cancer of the colon and rectum[5, 6, 7] is the third leading cause of cancer in males and fourth leading cause of cancer in females. The frequency of colorectal cancer varies around the world. It is common in the western world and is rare in Asia and Africa. In countries where the people have adopted western diets, the incidence of colorectal cancer is increasing. Factors that increase a person's risk of colorectal cancer include high fat intake, a family history of colorectal cancer and polyps, the presence of polyps in the large intestine, and chronic ulcerative colitis.

The various receptor targets for cancer are as follows:

- Mammalian target of rapamycin receptor (mTOR)
- Epidermal growth factor receptor (EGFR)
- Platelet-derived growth factor receptor (PDGF)
- Adenosine receptor
- Estrogen receptor
- G-protein-coupled receptors
- Chemokine receptors
- Toll-like receptors
- Cyclin-dependent kinase receptors (CDK)
- Cannabinoid receptors
- Fibroblast growth factor receptors (FGF)
- Insulin-like growth factor receptors (IGF)
- Hepatocyte growth factor receptors (HGF)

- Interferon receptors (IFN)

The mammalian target of rapamycin (mTOR) [8, 9, 10, 11] is an intacellular kinase that controls the production of several proteins through its phosphorylation of translational machinery. mTOR-activated proteins promote several hallmarks of cancer such as cell growth and proliferation, angiogenesis, and bioenergetics. Since mTOR acts as a neoplastic switch that is frequently turned on by many mutations found in cancer, inhibition of mTOR may offer a promising new strategy for cancer therapy.

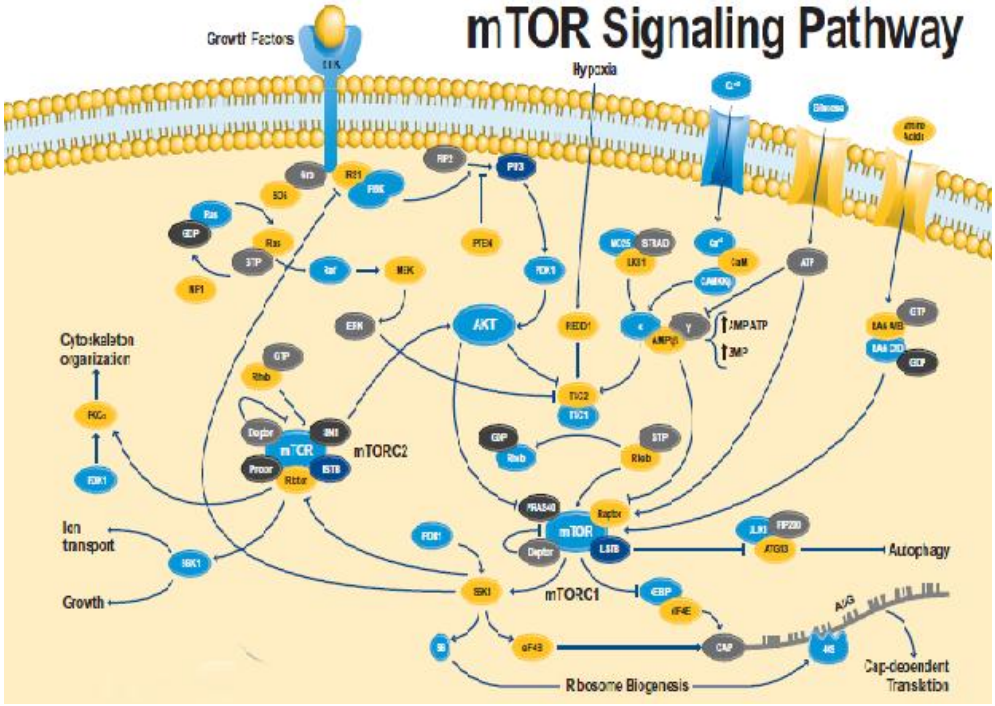


Fig.2 mTOR signaling pathway

The mammalian target of rapamycin (mTOR), also known as FKBP 2-rapamycin associated protein (FRAP), a phosphatidylinositol 3-kinase (PI3K) related serine/threonine kinase. The pathway in which it plays a prominent part regulates the growth, proliferation, motility and survival of cells and also angiogenesis. This central regulation of cell growth and proliferation is activated by growth factor/mitogenic stimulation activation of the

phosphatidylinositol 3-kinase (PI3K)/AKt signaling pathway[12,13,14], one of the most frequently dysregulated pathways in cancer. This pathway has been shown to cooperate in prostate cancer progression and the transition to androgen-independent disease. Rapamycin, a known mTOR inhibitor is a bacterial product that was originally of interest for its antifungal properties. It was subsequently found to have immunosupressive and antiproliferative properties. While it was being tested as an immunosuppressive agent to prevent organ rejection in transplant patients, the drug rapamycin was also discovered to have anti tumor properties. Rapamycin shows promise against few types of cancers particularly mantle cell lymphoma, endometrial cancer, and renal cell carcinoma. The known mTOR inhibitor rapamycin and its analogues (RAD001, CCI-779, and AP23573) bind to the FKBP12/rapamycin complex binding domain (FRB) and suppress the signaling to the downstream targets p70S6K and 4E-BP1. The potent but non-specific inhibitors of PI3K, LY294002 and wortmannin, have also been shown to inhibit the kinase function of mTOR but it inhibits by targeting the catalytic domain of protein. Recently it has been shown that mTOR exists in two complexes. mTORC1, a rapamycin sensitive complex signaling to p70S6K and 4E-BP1 and mTORC2, a rapamycin insensitive complex that signals to AKt. Inhibition of mTORC1 alone can block a desirable negative feedback mechanism, thereby causing an increase of PI3K/AKt signaling and reducing the effectiveness of the inhibitors. This negative feedback mechanism can be restored by inhibiting mTORC2. Therefore it is proposed that direct targeting of the kinase domain of mTOR would inhibit the signaling through both mTORC1 and mTORC2 and that such a compound would exhibit a different pharmacology compared with rapamycin. Since there is

no available crystal structure of mTOR, mTOR homology model was built based on the X-ray crystal structure of the closely related protein PI3Kγ.

1.1. *Homology modeling:* [15]

The ultimate goal of protein modeling is to predict a structure from its sequence with an accuracy that is comparable to the best results achieved experimentally. Protein modeling is the only way to obtain structural information if experimental techniques fail. Many proteins are simply too large for NMR analysis and cannot be crystallized for X-ray diffraction.

Homology modeling is a multistep process that can be summarized in seven steps:

1. Template recognition and initial alignment
2. Alignment correction
3. Backbone generation
4. Loop modeling
5. Side-chain modeling
6. Model optimization
7. Model validation

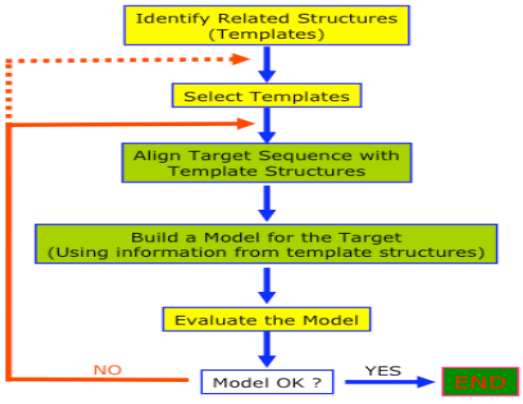


Fig. 3 Homology modeling

Every homology model contains errors. The number of errors (for a given method) mainly depends on two values:

- The percentage sequence identity between template and target. If it is greater than 90%, the accuracy of the model can be compared to crystallographically determined structures, except for a few individual side chains. From 50% to 90% identity, the root mean square (rms) error in the modeled coordinates can be as large as 1.5 A°, with considerably larger local errors. If the sequence identity drops to 25%, the alignment turns out to be the main bottleneck for homology modeling, often leading to very large errors.
- The number of errors in the template.

1.2. *Drug discovery:* [16, 17, 18]

Medicinal chemistry blends synthetic chemistry, molecular modeling, computational biology, structural genomics and pharmacology to discover and design new drugs, and investigate their interaction at the molecular, cellular and whole-animal level.

It combines empirical knowledge from the structure-function relationships of known drugs with rational designs optimizing of known drugs with rational designs optimizing the physiochemical properties of drug molecules.

The process of drug discovery involves the identification of candidate molecules, synthesis, characterization, screening for therapeutic efficacy and toxicity studies. The process of finding a new drug against a chosen target for a particular disease usually involves high-throughput screening (HTS), wherein large libraries of chemicals are tested for their ability to modify the target.

Drug discovery and development can broadly follow two different paradigms- Physiology-based drug discovery and Target-based discovery. The main difference between these two paradigms lies in the time point at which the drug target is actually identified.

Physiology-based drug discovery follows physiological readouts, for example, the amelioration of a disease phenotype in an animal model or cell-based assay. A purely physiology-based approach would initially forgo target identification/validation and instead jump right into screening. Identification of drug target and the mechanism of action would follow in later stages of the process by deduction based on the specific pharmacological properties of lead compounds.

By contrast, the road of target-based drug discovery begins with identifying the function of a possible therapeutic target and its role in disease.

One way to find promising drug candidates is to investigate how the target protein interacts with randomly chosen compounds. This is done by using compound libraries which can contain more than a million synthetic and natural compounds. These libraries are then tested against the target protein. This is most often done in so called high-throughput screening facilities. The most promising compounds obtained from the screening process are called hits-these are the compounds showing binding activity. Some of these hits are then promoted to lead compounds-candidate structures which are further refined and modified in order to achieve more favourable interactions and less side-effect.

Advances in computing power and in structure determination by X-ray crystallography and NMR have made computer-aided drug design (CADD) and structure-based drug design (SBDD) essential tools for drug discovery.

The main advantages of computational methods over wet-lab experiments are as follows:

- Low costs, no compounds have to be purchased externally or synthesized by a chemist.
- It is possible to investigate compounds that have not been synthesized yet.
- Conducting high-throughput screening (HTS) experiments is expensive and virtual screening (VS) can be used to reduce the initial number of compounds before using high-Throughput Screening (HTS) methods.
- Huge chemical search space. The number of possible virtual molecules available for VS is much higher than the number of compounds presently available for HTS.

1.2.1. CADD of lead compounds:

A detailed knowledge of a target binding site significantly aids in the design of novel lead compounds intended to bind with that target. In cases, where enzymes or receptors can be crystallized, it is possible to determine the structure of the protein and its binding site by X-ray crystallography. Molecular modeling software can then be used to study the binding site, and to design molecules which will fit and bind to the site-de novo drug design.

In some cases, the enzymes or receptor cannot be crystallized and so X-ray crystallography cannot be carried out. However, if the structure of an analogous protein has been determined, this can be used as the basis for generating a computer model of the protein (Homology Modeling). Homology Modeling relies on the identification of one or more known protein structures likely to resemble the structure of the query sequence, and on the production of an alignment that maps residues in the query sequence to residues in the

template sequence. The sequence alignment and template structure are then used to produce a structural model of the target. The quality of the model is dependent on the quality of the sequence alignment and template structure.

Lipinski's rule of five[19] is a rule of thumb to evaluate drug likeness or determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans. The rule is important for drug development where a pharmacologically active lead structure is optimized-step-wise for increased activity and selectivity, as well as drug-like properties as described by Lipinski's rule.

- a molecular weight less than 500
- no more than 5 hydrogen bond donor groups
- no more than 10 hydrogen bond acceptor groups
- a calculated log P value less than +5 (log P is a measure of a drug's hydrophobicity)

1.2.2. *Molecular docking:* [20, 21]

Molecular docking programs try to predict how a drug candidate binds to a protein target without performing a laboratory experiment. Molecular docking software consists of two core components.

- A search algorithm (sometimes called an optimization algorithm). The search algorithm is responsible for finding the best conformations of the ligand and protein system. A conformation is the position and orientation of the ligand relative to the protein. In flexible docking, a conformation also contains

information about the internal flexible structure of the ligand and in some cases about the internal flexible structure of the protein. Since the number of possible conformations is extremely large, it is not possible to test all of them, therefore sophisticated search techniques have to be applied. Examples of some commonly used methods are Genetic Algorithms and Monte Carlo Simulations.

- An evaluation function (sometimes called a score function). This is a function providing a measure of how strongly a given ligand will interact with a particular protein. Energy force fields are often used as evaluation functions. These force fields calculate the energy contribution from different terms such as the known electrostatic forces between the atoms in the ligand and in the protein forces arising from deformation of the ligand, pure electron-shell repulsion between atoms and effect from the solvent in which the interaction takes place.

1.2.3. Pharmacophore mapping:

Pharmacophore mapping is a geometrical approach. A pharmacophore can be thought as a 3D model of characteristic features of the binding site of the investigated protein. It can also be thought of as a template, a partial description of a molecule where certain blanks need to be filled. Like QSAR models, pharmacophores can be built without knowing the structure of the target. This can be done by extracting features from compounds which are known experimentally to interact with the target in question. Afterwards, the derived pharmacophore model can be used to search compound databases (libraries) thus screening for potential drug candidates that may be of interest.

Identifying 3D pharmacophore is relatively easy for rigid cyclic structures. With more flexible structures, it is not so straightforward because the molecule can adopt a large number of shapes or conformations which place the important binding groups in different positions relative to each other. Normally only one of these conformations is recognized and bound by the binding site. This conformation is known as the active conformation.

In order to identify the 3D pharmacophore, it is necessary to know the active conformation. There are various ways in which this might be done. Rigid analogues of the flexible compound could be synthesized and tested to see whether activity is retained. Alternatively, it may be possible to crystallize the target with the compound bound to the binding site. X-ray crystallography could then be used to identify the structure of the complex as well as the active conformation of the bound ligand.

1.2.4. Lead optimization:

Lead optimization is the complex, non-linear process of refining the chemical structure of a confirmed hit to improve its drug characteristics with the goal of producing a preclinical drug candidate. This stage frequently represents the bottleneck of a drug discovery program.

Once the important binding groups and pharmacophore of the lead compound have been identified it is possible to synthesize analogues that contain the same pharmacophore. Very few lead compounds are ideal. Most are likely to have low activity, poor selectivity, and significant side effects. They may also be difficult to synthesize, so there is an advantage in finding analogues with improved properties.

The following strategies are used to optimize the interactions of a drug with its target in order to gain higher activity and selectivity.

- Variation of substituents
- Extension of the structure
- Chain extension/contraction
- Ring expansion/contraction
- Ring variations
- Ring fusions
- Isosteres and Bioisosteres
- Simplification of the structure
- Rigidification of the structure
- Conformational blockers

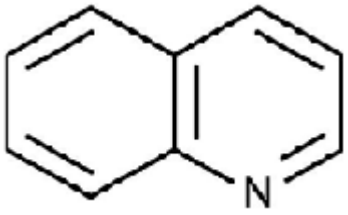
1.3. *Quinoline:* [22]

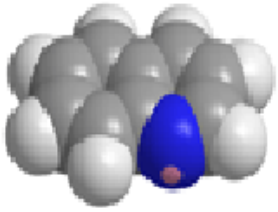
Heterocycles form by far the largest of classical divisions of organic chemistry and are of immense importance biologically and industrially. The majority of pharmaceuticals and biologically active agrochemicals are heterocyclic. One striking structural features inherent to heterocycles, which continue to be exploited to great advantage by the drug industry lies in their ability to manifest substituents around a core scaffold in defined three dimensional representations.

For more than a century, heterocycles have constituted one of the largest areas of research in organic chemistry. Between them nitrogen and sulfur containing heterocyclic compounds have maintained the interest of researches through decades of historical development of organic synthesis.

Quinoline is a heterocyclic aromatic nitrogen compound characterized by a double ring structure that contains a benzene ring fused to pyridine at two adjacent carbon atoms.

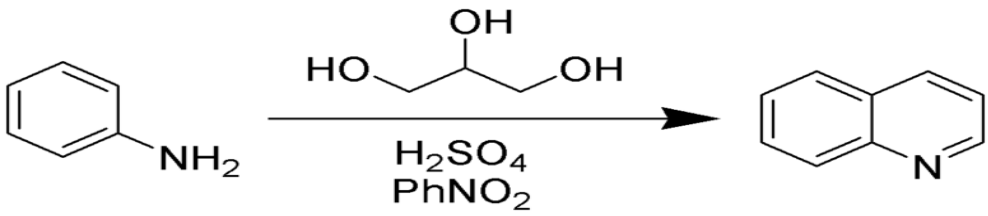
Its principal use is as a precursor to 8-hydroxyquinoline, which is a versatile chelating agent and precursor to pesticides. Its 2- and 4-methyl derivatives are precursors to cyanine dyes. Oxidation of quinoline affords quinolinic acid (pyridine-2, 3-dicarboxylic acid), a precursor to the herbicide sold under the name "Assert". Like other nitrogen heterocylic compounds, such as pyridine derivatives, quinoline is often reported as an environmental contaminant associated with facilities processing oil shale or coal. Owing to high water solubility quinoline has significant potential for mobility in the environment, which may promote water contamination. Fortunately, quinoline is readily degradable by certain microorganisms, such as *Rhodococcus* species Strain Q1, which was isolated from soil and paper mill sludge.





Quinoline was first extracted from coal tar in 1834 by Friedlieb Ferdinand Runge. Coal tar remains the principal source of commercial quinoline. It can be synthesized by various methods.

E.g. Skraup Synthesis



REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Cancer research is the intense scientific effort to understand disease processes and discover possible therapies. Though many diseases (such as heart failure) may have a worst prognosis than most cases of cancer, cancer is the subject of widespread fear. In this literature review few works are listed here in support of cancer research.

B.Ganesh et.al, [3] **(2009)** A case-control study on diet and colorectal cancer from Mumbai, India.

Rajaraman Swaminathan et.al, [4] (2009) reported the Cancer pattern and survival in a rural district in South India.

Suzanne Hector et.al, [7] **(2009)** reported Apoptosis signaling proteins as prognostic biomarkers in colorectal cancer: A review

J.Cardoso et.al, [5] **(2007)** reported the Expression and genomic profiling of colorectal cancer.

H.E.Blum [6] **(1995)** Colorectal Cancer: Further population screening for early colorectal cancer.

2.2. Being a key regulator in cell growth and proliferation, mTOR's deregulation is associated with human diseases including cancer and diabetes. The following literatures are listed here in support of cancer.

Carlos Garcia-Echeverria [11] **(2010)** Allosteric and ATP-competitive kinase inhibitors of mTOR for cancer treatment.

Margit Rosner et.al, [13] **(2008)** The mTOR pathway and its role in human genetic diseases.

JB Easton and PJ Houghton [10] **(2006)** mTOR and cancer therapy.

Laura Asnaghi et.al., [8] **(2004)** mTOR: a protein kinase switching between life and death.

Jie Chen, Yimin Fang [15] **(2002)** A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling.

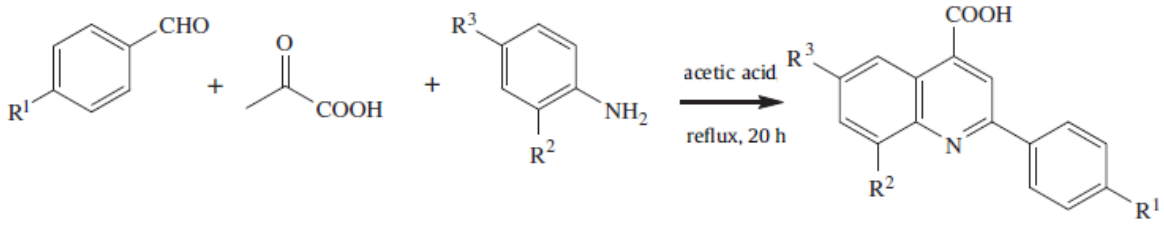
2.3. Review related to modeling of target protein for drug design:

Karunakar tanneeru et.al, [24] **(2011)** Ligand-based 3-D pharmacophore generation and molecular docking of mTOR kinase inhibitors.

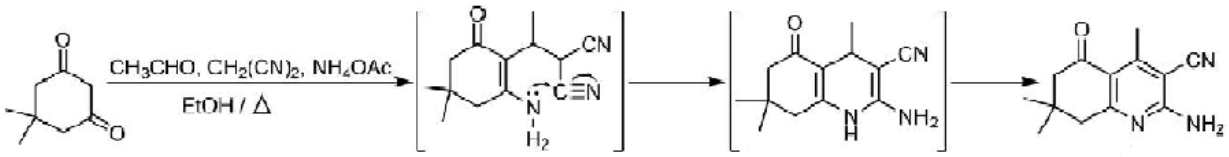
2.4. The synthetic versatility of quinoline allows us to generate more diverse analogues. Of the several work of quinoline, few of them are listed here in support of their different synthesis.

K.D. Thomas et.al, [38] **(2011)** Design, synthesis and docking studies of new quinoline-3-carbohydrazide derivatives as antitubercular agents.

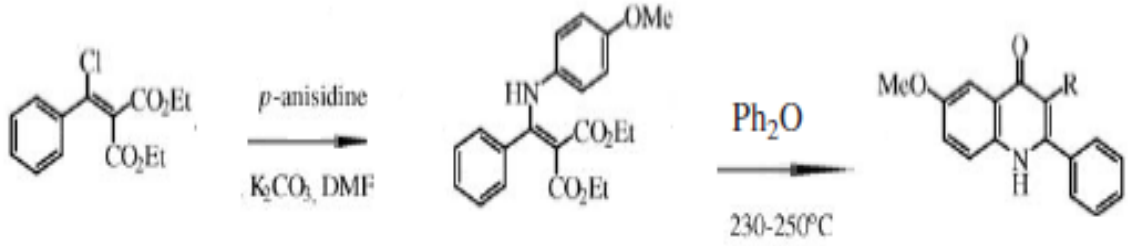
Afshin Zarghi et.al, [37] **(2010)** reported the Design, synthesis, and biological evaluation of ketoprofen analogs as potent cyclooxygenase-2 inhibitors.



Mostafa M. Ghorab et.al, [35] **(2009)** Design, synthesis and anticancer evaluation of novel tetrahydroquinoline derivatives containing sulfonamide moiety.

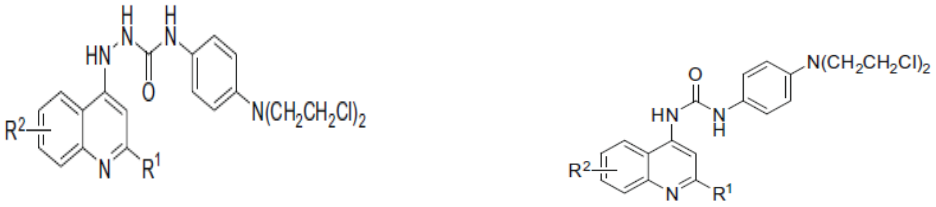


Yue-Ling Zhao et.al, [36] **(2005)** reported the Synthesis and cytotoxic evaluation of certain 4-anilino-2-phenylquinoline derivatives.

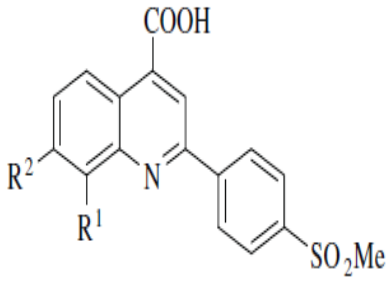


2.5. Of the several works of quinoline, few of them are listed here in support of their wide variety of biological activity.

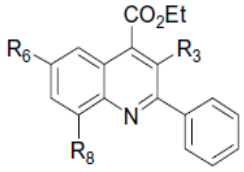
Rajesh Kakadiya et.al, [47] **(2010)** Potent DNA-directed alkylating agents: Synthesis and biological activity of phenyl N-mustard–quinoline conjugates having a urea or hydrazinecarboxamide linker.

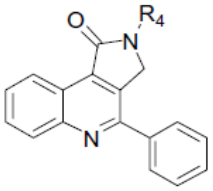


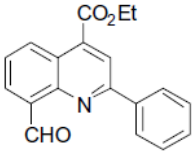
Afshin Zarghi et.al, [48] **(2009)** reported the Synthesis and biological evaluation of new 4-carboxyl quinoline derivatives as cyclooxygenase-2 inhibitors.



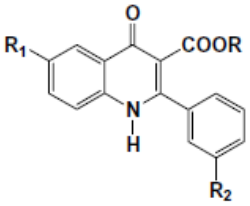
Andrew N. Boa et.al, [49] **(2005)** reported the Synthesis of brequinar analogue inhibitors of malaria parasite dihydroorotate dehydrogenase.

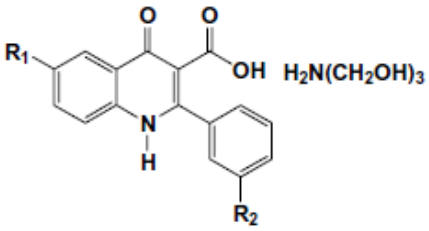






Ya-Yun Lai et.al, [50] **(2005)** reported the Synthesis and biological relationships of 3',6-substituted 2-phenyl-4-quinolone-3-carboxylic acid derivatives as antimitotic agents.





2.6. Reviews realted to cytotoxic studies using MTT assay:

Ichiro Miki et.al, [46] **(1993)** Simple colorimetric cell-cell adhesion assay using MTT stained leukemia cells.

Arjan A.van de Loosdrecht et.al, [45] **(1991)** Cell mediated cytotoxicity against U 937 cells by human monocytes and macrophages in a modified colorimetric MTT assay.

AIM AND OBJECTIVE

3. AIM AND OBJECTIVE

Quinoline compounds are widely used as “parental” compounds to synthesize molecules with medical benefits, especially with anti-malarial and anti-microbial activities. Certain quinoline-based compounds also show effective anticancer activity. This broad spectrum of biological and biochemical activities has been further facilitated by the synthetic versatility of quinoline, which allows the generation of a large number of structurally diverse structures.

The objective of the current study includes:

- Fragment identification
- Evaluation of molecules against specific protein target using In-silico methods.
- Synthesis of following compounds
    - (6-chloro-2-phenylquinolin-4-yl)(1H-imidazol-1-yl)methanone [C_1]
    - (6-chloro-2-phenylquinolin-4-yl)(piperidin-1-yl)methanone[C_2]
    - 6-nitro-2-phenyl-N-(pyridine-2-yl)-1,2,3,4-tetrahydroquinoline-4-carboxamide [C_3]
    - {2-[4-(dimethyl amino)phenyl]-6-nitro-1,2,3,4-tetraquinolin-4-yl}(piperidin-1-yl)methanone [C_4]
    - {2-[4-(dimethyl amino)phenyl]-6-nitro-1,2,3,4-tetraquinolin-4-yl}(1H-imidazol-1-yl)methanone [C_5]
- Characterization of the above synthesized compounds by UV Spectroscopy, IR Spectroscopy, NMR Spectroscopy and MASS Spectrometry.
- Cytotoxic studies of the synthesized compounds using MTT assay technique
- Toxicological evaluation

MATERIALS & METHODS

4. MATERIALS AND METHODS

4.1. *Homology modeling:* [23]

Homology model of mTOR kinase domain was built using Accelrys® discovery studio. Modeler algorithm was used to generate the 3D structure of mTOR based on the crystal structure of PI3K gamma (PDB ID: 3S2A). The structure has a resolution of 2.5 Å and exists in complex with a quinoline inhibitor. The c-terminal region of human mTOR protein sequence was taken from uniprot database (P42345). The sequence alignment was carried out using the ClustalW program to identify homologous regions between the two proteins. The catalytic domain of mTOR and PI3K gamma shows maximum of 45% similarity. After the identification of structurally conserved and variable regions, restraints, distances and dihedral angles were extracted from the template structure and applied to mTOR. Stereochemical restraints, viz., bond length and bond angle preferences, were obtained from the molecular mechanics force field CHARMM. The quinoline inhibitor from the crystal structure was extracted and transferred to mTOR homology model for further guidance in docking studies. mTOR homology model was further refined using 600 ps MD simulations in explicit water. Minimization was carried out using the consistent valence force field (CVFF) with a van der Waals cutoff of 9.5 ˚A and a distance-dependent dielectric constant of 1×r. One thousand steps of steepest descents were performed followed by 1000 steps of conjugate gradients until the root mean square (RMS) gradient reached a value of less than 0.001kcal/mol/Å. The homology models were each solvated with a 10 ˚A water layer and optimized using MD simulations for 2 ns at a 300 K temperature. The

quality of the model was assessed by PROCHECK. The model was evaluated by a Ramachandran plot and found that 97% of the residues are in favorable region.

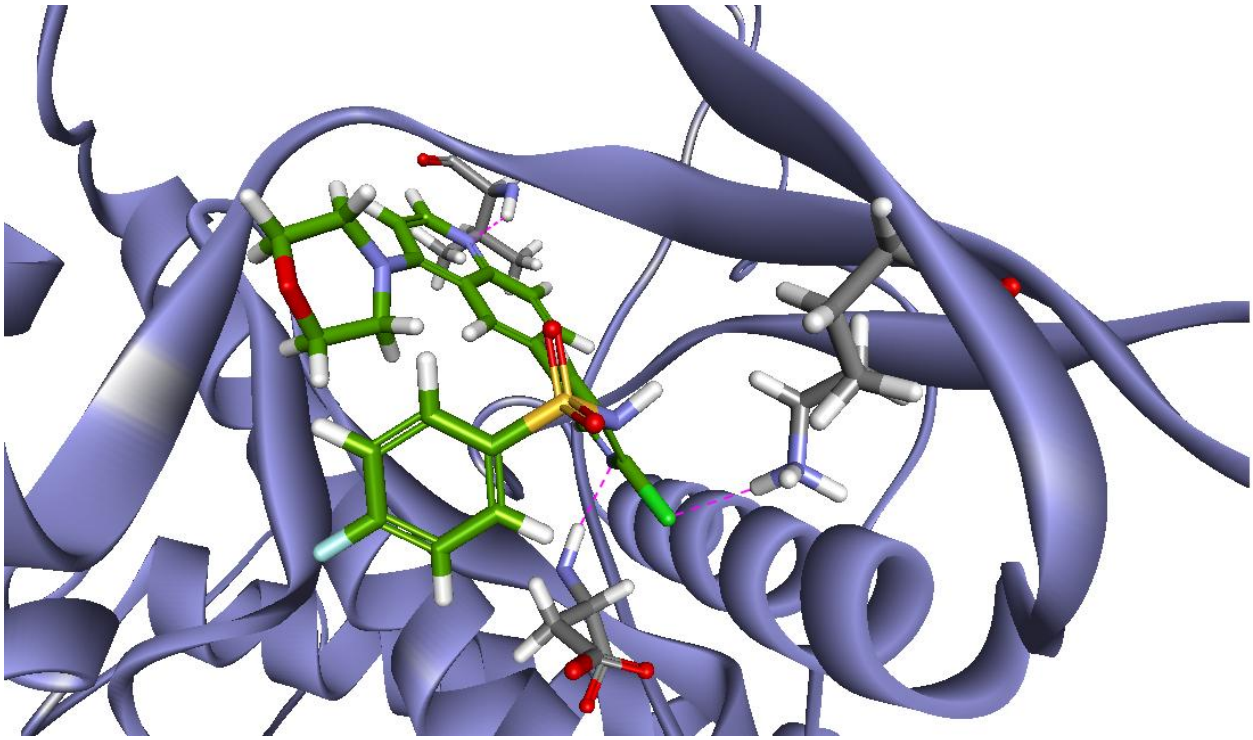


Fig.4 Homology model of human mTOR in complex with ligand extracted from PI3K gamma. Dotted lines shows the interactions with the protein with Asp 177, Lysine 97 and Valine 60

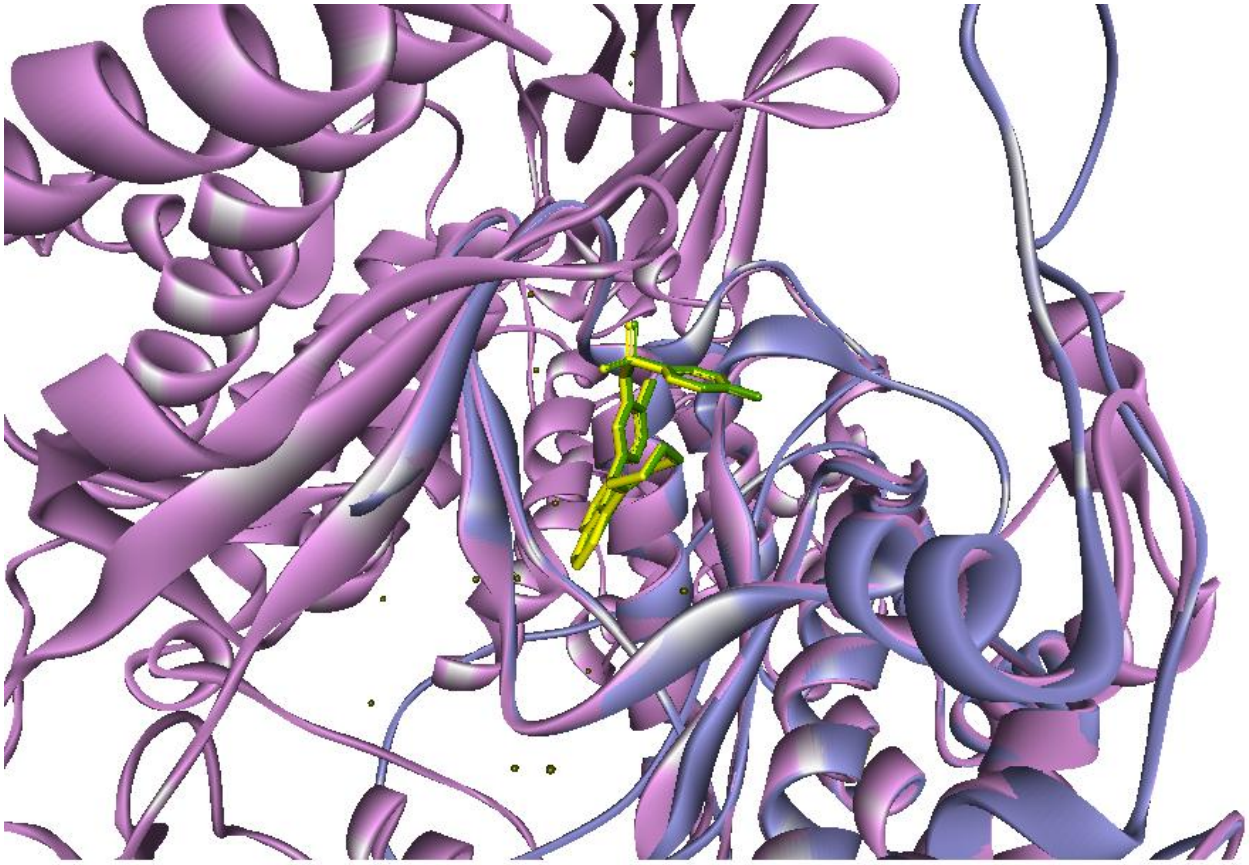


Fig.5 Structural overlay of human mTOR with PI3K gamma in complex with ligand.

Blue – mTOR; Pink – PI3K gamma

4.2.*Drug design:* [24,25]

A drug is a small molecule ligand that binds to a specific protein which either increases its activity (an agonist) or decrease/block its activity (an antagonist). One way to find promising drug candidates is to investigate how the target protein interacts with randomly chosen compounds. Drug designing basically of two types namely ligand based approach or receptor based approach.

- Ligand-based drug design (or indirect drug design) relies on knowledge of other molecules that bind to the biological target of interest. These other molecules may be used to derive a pharmacophore model that defines the minimum necessary structural characteristics a molecule must possess in order to bind to the target.
- Structure-based drug design (or direct drug design) relies on knowledge of the three dimensional structure of the biological target obtained through methods such as x-ray crystallography or NMR spectroscopy. If an experimental structure of a target is not available, it may be possible to create a homology model of the target based on the experimental structure of a related protein.

4.2.1. *Pharmacophore studies using catalyst:* [26, 27, 28]

Pharmacophore means “a molecular framework that carries (phoros) the essential features responsible for a drugs (pharmacon) biological activity”.

Catalyst® 4.11 from accelrys:

For Rational design of small molecules as drug candidates using 3D pharmacophore and shape-based models, and to suggest potentially active compounds suitable for synthesis and biological testing. The typical features are hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), hydrophobic (HY), hydrophobic aliphatic, hydrophobic aromatic, positive ionizable, negative ionizable and ring aromatic(RA).

Chemical feature based models from Catalyst® 4.11:

- HipHop

  Generates a set of common feature pharmacophore models from set of compounds known to be active (No activity data) at a specific target.

- HypoGen

  Develop SAR hypothesis models from a set of molecules for which activity values (IC50 or Ki) on a given biological target are known.

- HypoRefine

  Permits consideration of exclusion volumes in pharmacophore-based 3D QSAR optimization. The result is to better model predictivity where biological activity is determined by considerations of molecular shape.

- Exclusion volume

  An excluded volume can be added to a hypothesis (or to a template molecule) to specify one or more spherical spaces that must not contain any atoms or bonds. An exclusion volume can represent a region in space that might

impinge sterically on a receptor. An exclusion volume can be interpreted as a geometrical constraint, and this is how it is treated in catalyst®.

- Compare/Fit

  Provides the ability to fit compounds and hypotheses, and determine their degree of similarity, both geometrically and functionally. In database search, COMPARE fits the original hypothesis onto the hit molecules obtained from the search and score are calculated according to the geometrical fit.

- Cost parameters
  - Fixed cost represents the simplest possible hypothesis (initial) that fits the data perfectly.
  - Null hypothesis-It is the costs when each molecule estimated as mean activity. It acts like a hypothesis with no features.
  - Error cost-The bits needed to describe the error in the leads. It increases as the RMS difference between estimated and measured activities for training set molecules increases.
  - Weight cost-The bits required to describe the feature weights.
  - Configuration cost-The bits required to describe the types and relative positions of the features in the hypothesis. A fixed cost that depends on the

    -main assumption made by HypoGen is that an active molecule

    -should map more features than an inactive molecule

-complexity of the hypothesis space being optimized

HipHop model (Qualitative):

- Identifies configurations or three-dimensional spatial arrangements of chemical features that are common to molecules in a training set.
- HipHop matches the chemical features of a molecule against drug candidate molecules or searches of 3D databases.
- The resulting hypotheis can be used to iteratively search chemical databases to find new lead candidates.

HypoGen model(Quantitative):

- Creates SAR hypothesis models from a set of molecules for which activity values are known.
- Selects pharmacophore that are common among the active compounds but not among the inactive compounds, then optimizes the pharmacophores using simulated annealing.
- Best pharmacophores can be used to predict the activity of unknown compounds or to search for new possible leads contained in 3D chemical databases.
- Selection of training set is very important, since the hypotheses are derived directly from the information in the training set.

  The quality of HypoGen model are best described in terms of cost analysis.

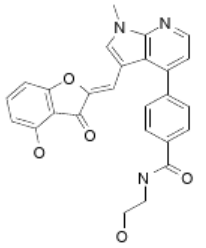
4.2.2. Data set:

Pharmacophore modeling correlates activities with the spatial arrangement of various chemical features in a set of active analogues. The compounds in this study were collected from a series of mTOR inhibitors published in recent years, considering both structural diversity and wide coverage of the activities. A set of 297 human mTOR inhibitors with an activity range (IC_{50}) 0.0016-11000 nM was selected. This initial group was then divided into the training set and test set. The training set of 24 molecules was designed to be structurally diverse with a wide activity range. Molecules with K_i, ED_{50}, EC_{50} and other activity type values were ignored and not considered for modeling studies. The training set molecules play a critical role in the pharmacophore generation process and the quality of the resultant pharmacophore models relies solely on the training set molecules. The test set of remaining 273 molecules is designed to evaluate predictive ability of the resultant pharmacophore. Highly active, moderately active, and inactive compounds were added to the training set to obtain critical information on pharmacophoric requirements for mTOR inhibition. The molecules selected as the training set are given in fig.6 and a few molecules from test set are given in fig.7. This training set was then used to generate quantitative pharmacophore models. While generating a quantitative model, a minimum of 0 to a maximum of 4 features involving hydrophobic acceptor (HBA), hydrophobic donor (HBD), hydrophobic aliphatic and ring aromatic (RA) were selected and used to build a series of hypotheses using a default uncertainity value of 3. The quality of HypoGen models is best described in catalyst user guide in terms of fixed cost, null cost and total cost and

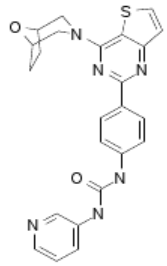
other statistical parameters. According to which, a large difference between the fixed cost and null cost, and a value of 40-60 bits for the unit of cost would imply a 75-90% probability for experimental and predicted activity correlation. In general, pharmacophore models should be statistically significant, predict the activity of molecules accurately, and retrieve active compounds from a database. The derived pharmacophore models were validated using a set of parameters including cost analysis, test set prediction, enrichment factors, and goodness of fit. HipHop and HypoGen modules within catalyst were then used to generate qualitative pharmacophore and quantitative pharmacophore models respectively.

4.2.3. Conformational analysis:

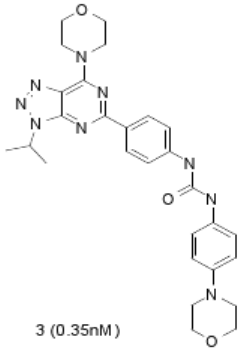
Molecular flexibility was explored by considering each compound as a collection of conformers representing different areas of the conformational space accessible to the molecule which in a given energy range. The conformational space of each compound was extensively sampled utilizing the Poling algorithm and CHARMM force field parameters with an energy threshold of 20Kcal/mol from the lowest energy level and a maximum of 250 conformers for compounds predicting their activities accurately and further performing a database searching.



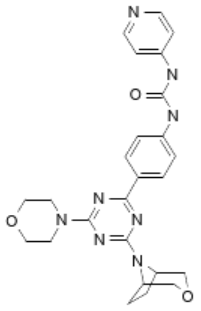
1 (0.0016nM)



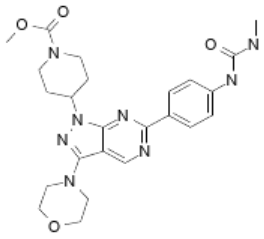
2 (0.29nM)



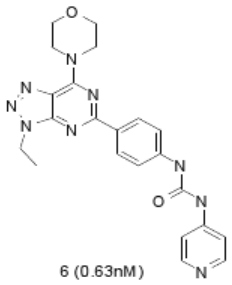
3 (0.35nM)



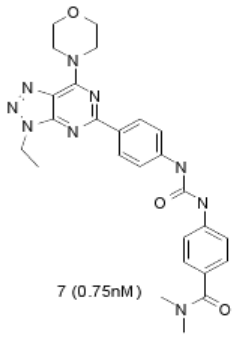
4 (0.42nM)



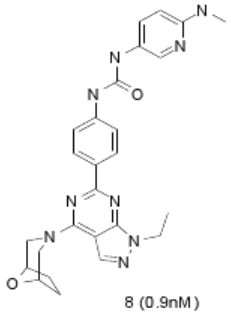
5 (0.46nM)



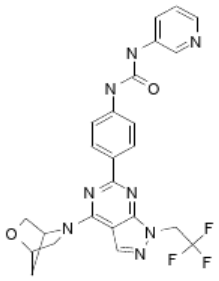
6 (0.63nM)



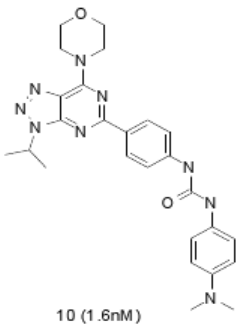
7 (0.75nM)



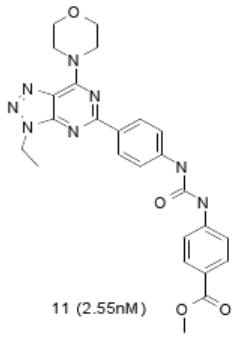
8 (0.9nM)



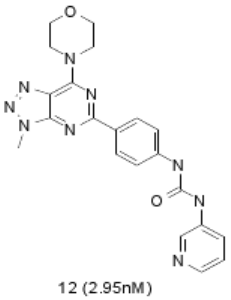
9 (1.2nM)



10 (1.6nM)



11 (2.55nM)



12 (2.95nM)

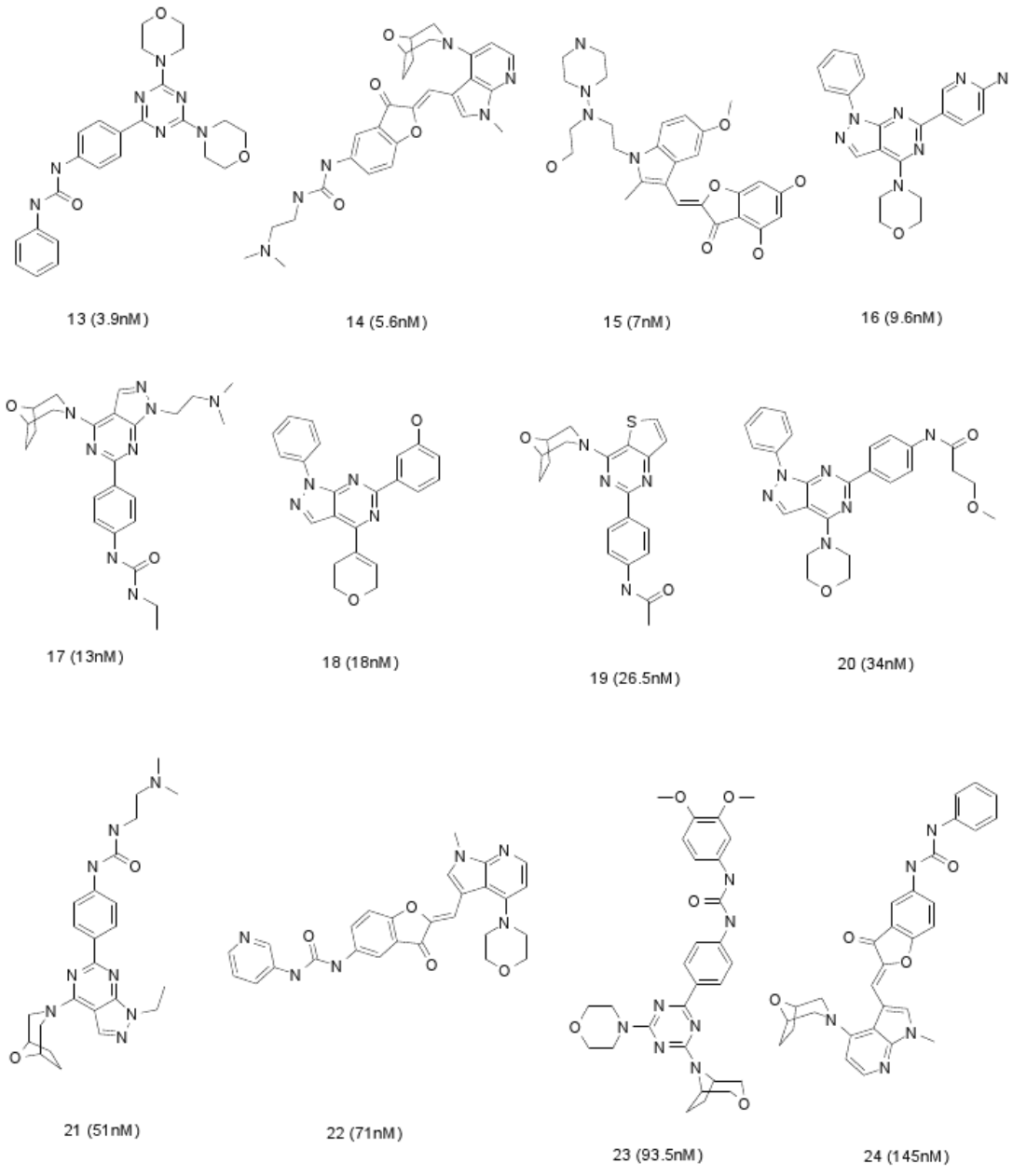


Fig. 6 Training set molecules

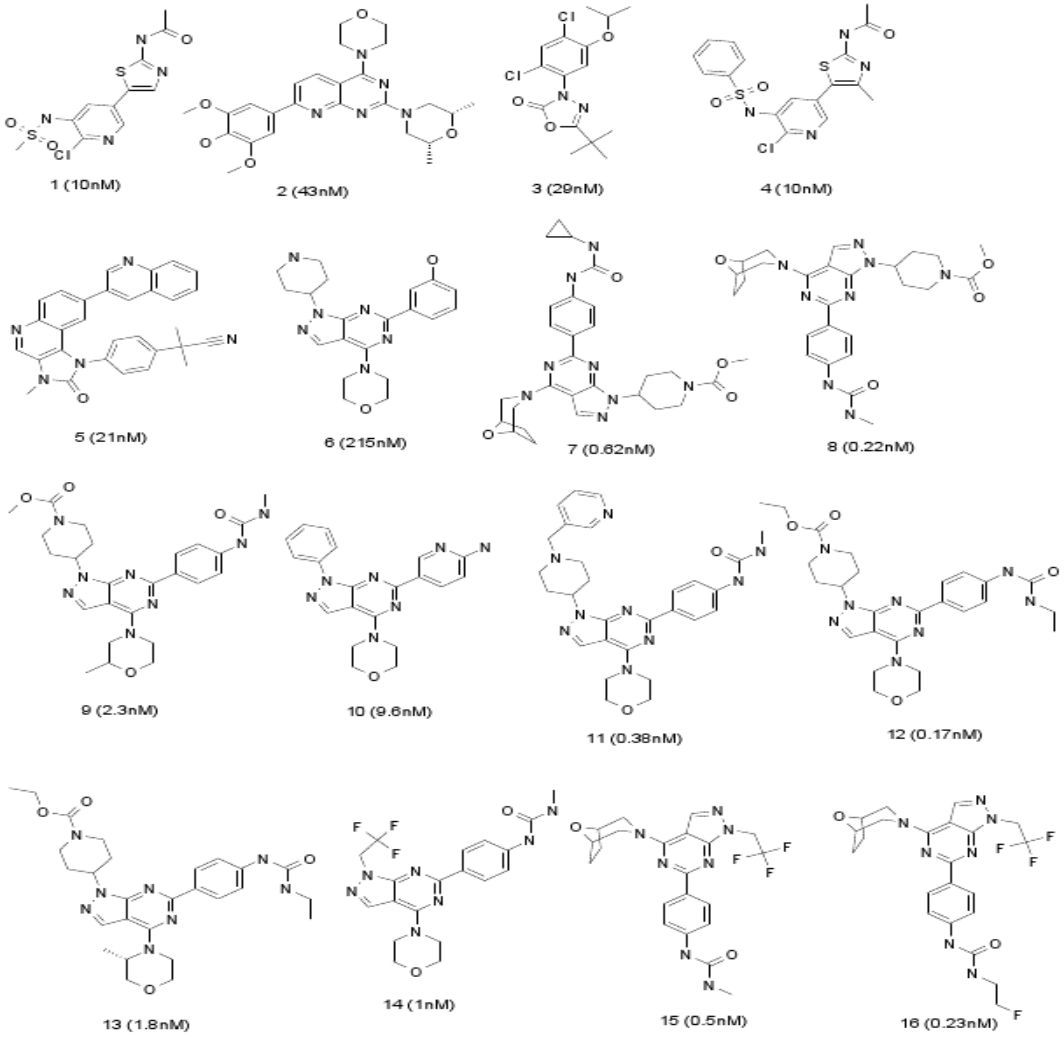


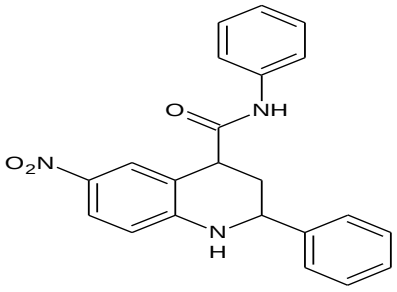
Fig. 7 Test set molecules

4.2.4. Database searching:

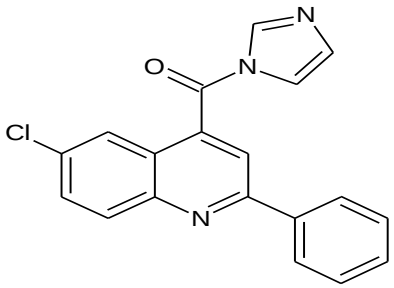
Virtual screening of chemical databases can serve the purpose of finding novel, potential leads suitable for further development. Database searching methodology provides the advantage that the retrieved compounds can be obtained easily for biological testing when compared to any de novo design methods. A molecule must fit on all the features of the pharmacophore model that is used as 3D query in database searching to be retained as hit. Two database searching options such as Fast/Flexible and Best/Flexible search are available in DS. Better results can be achieved using Best/Flexible search option during database screening.

4.2.5. ***Molecular Docking:*** [29,30]

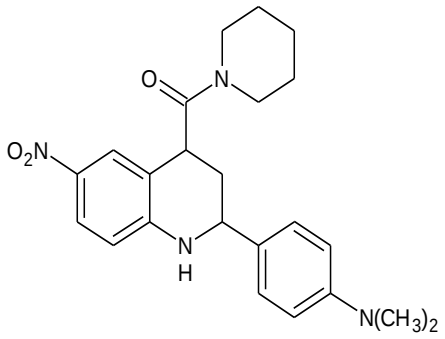
Molecular docking is a computationally intensive SBVS technique that generates and scores putative protein-ligand complexes according to their calculated binding affinities. Given the crystal structure of the target, molecular docking automatically samples ligand conformations and protein ligand interactions with a specified region of the protein surface. It has been successfully used for identifying active compounds by filtering out those that do not fit into the binding sites. In absence of the structural information of the target, a homology model can be constructed and used for the molecular docking. In this study, molecular docking was performed with a homology model of mTOR by the program Glide®.



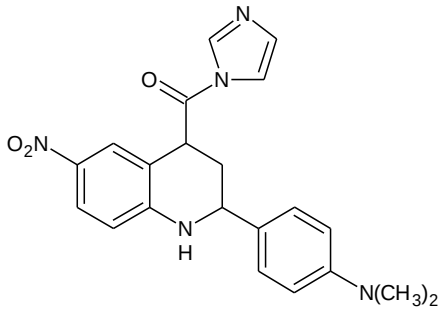
Compound 1
(G Score:-8.05)



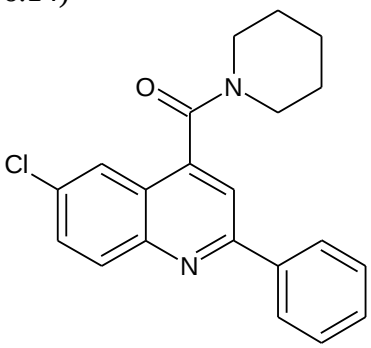
Compound 2
(G Score:-6.61)



Compound 3
(G Score:-6.14)



Compound 4
(G Score:-6.04)



Compound 5
(G Score:-4.85)

REWARDS:

Table 1:

Ligand	G Score	Lipophilic Evdw	PhobEn	PhobEn HB	PhobEn PairHB	H bond	Elect	Site map	Pi Cat	ClBr	Low Mw
1	-8.05	-3.71	-1.22	-1.5	0	-1.36	-0.82	-0.03	0	0	-0.19
2	-6.61	-3.97	-1.55	0	0	-0.9	-0.11	-0.4	0	0	-0.38
3	-6.14	-4.08	-1.7	0	0	-0.35	-0.13	-0.26	0	0	-0.13
4	-6.04	-4.67	-1.20	0	0	0	-0.23	-0.31	0	0	-0.19
5	-4.85	-3.75	-1.57	0	0	0	-0.06	-0.21	0	0	-0.33

PENALTIES:

Table 2:

Ligand	Penalties	HB Penal	PhobicPenal	RotPenal
1	0	0	0.59	0.21
2	0	0	0.49	0.21
3	0	0	0.37	0.15
4	0	0	0.42	0.16
5	0	0	0.88	0.19

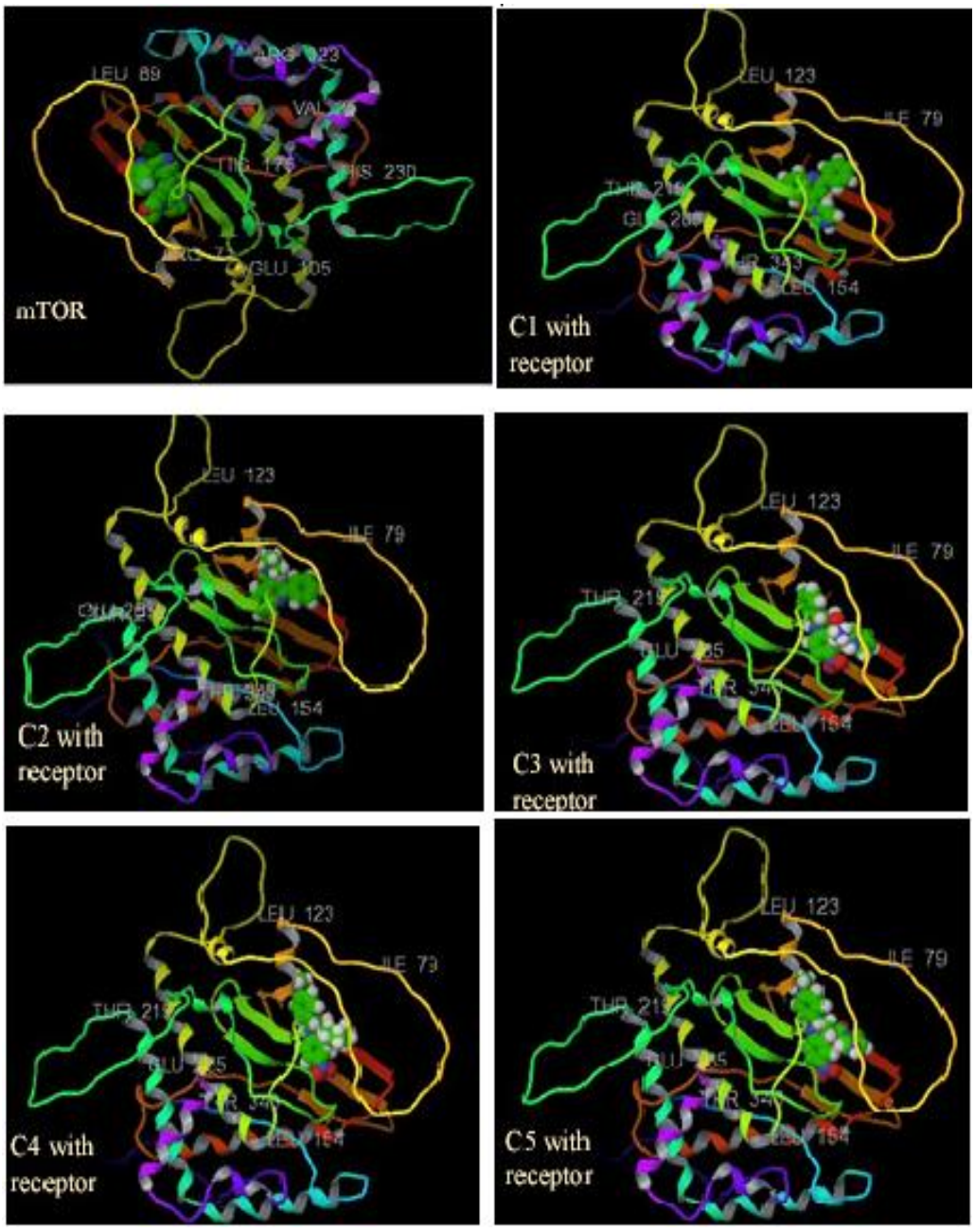


Fig. 8 Ligand binding with the receptor

4.2.6. *Lipinski's rule:* [31]

Lipinski's rule is to evaluate druglikeness of a chemical compound. Changes in the compound's bioactivity adjust to Lipinski's rule of five. The physical properties of the molecules were calculated using Molinspiration molecular properties calculator. The result values are given in table 3.

Table 3:

Compound No.	Log P	Mol.Wt	HBA	HBD	Rotatable Bonds	TPSA	Mol.vol.	No. of Violations
1	3.27	374.4	7	2	4	99.84	329.44	0
2	4.24	333.77	4	0	2	47.78	280.42	0
3	3.72	408.50	7	1	4	81.39	381.64	0
4	3.02	391.43	8	1	4	95.98	348.31	0
5	4.59	350.84	3	0	2	33.20	313.75	0

Log P- Partition Coefficient

Mol.Wt- Molecular Weight

HBA- Hydrogen Bond Acceptor

HBD- Hydrogen Bond Donor

TPSA- Total Polar Surface Area

Mol. Vol.- Molar Volume

4.3. *Synthesis:* [32, 33]

Step 1:

Pyruvic acid (22ml, 0.25 mol) in 200 ml of ethanol and benzaldehyde (24ml, 0.236 mol) were mixed and heated up to the boiling point. A solution of pure aniline (23ml, 0.248 mol) in 100 ml of ethanol was added. The addition was done for 1hr. The mixture was refluxed for about 3 hr and allowed to stand overnight.

Step 2:

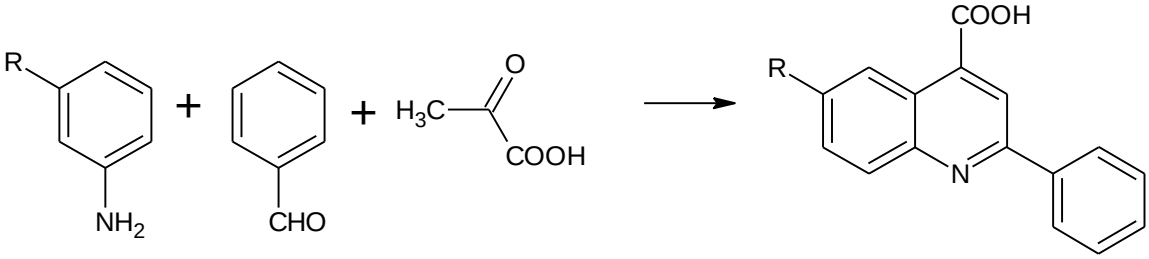
Prepared 2-phenyl-quinoline carboxylic acid (0.01 mole) and 15 ml of thionyl chloride were refluxed for 30 min. Unreacted thionyl chloride was removed by heating the reaction mixture on a water bath.

Step 3:

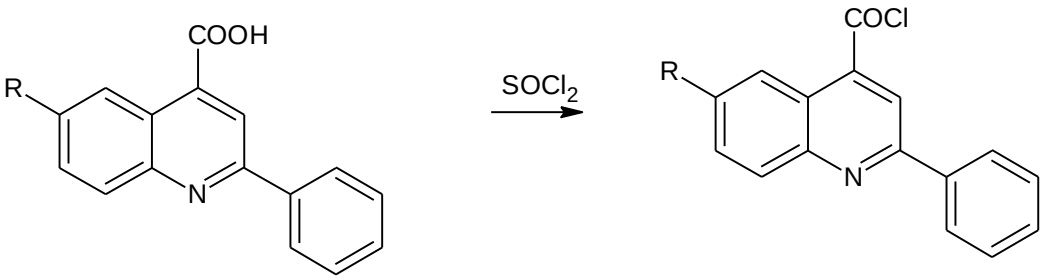
The above obtained acid chloride product was treated with 3 to 4 times of different amines and ethanol as reaction medium and stirred for 5 hrs. It was then added into cold water to get the precipitate, which was filtered and recrystallized from suitable solvent.

Scheme I:

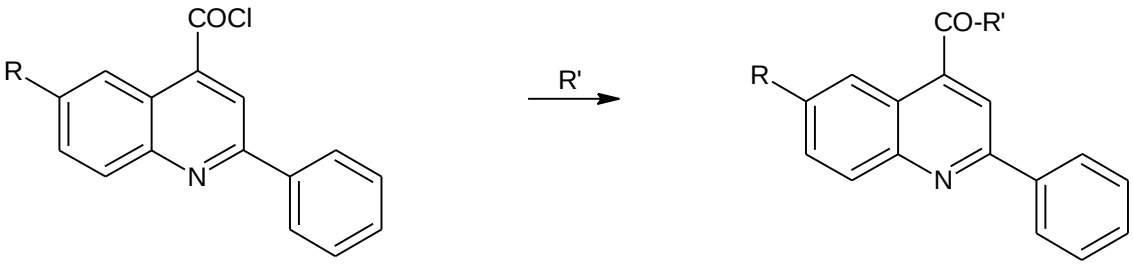
Step 1:



Step 2:



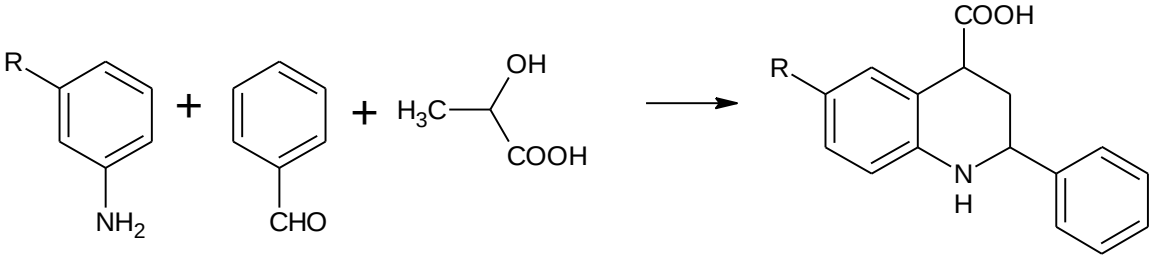
Step 3:



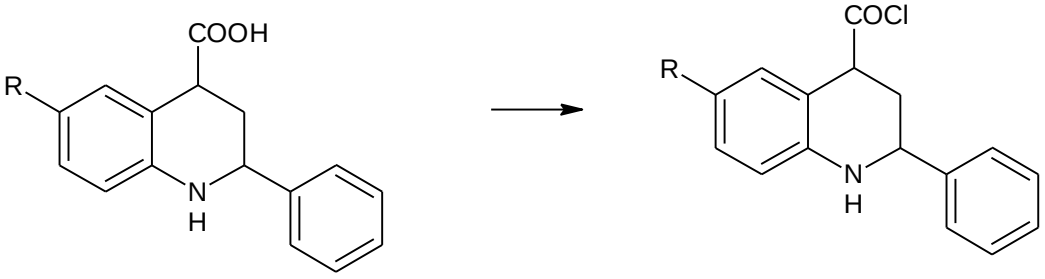
Where R=Cl; R'=Imidazole/Piperidine

SCHEME II:

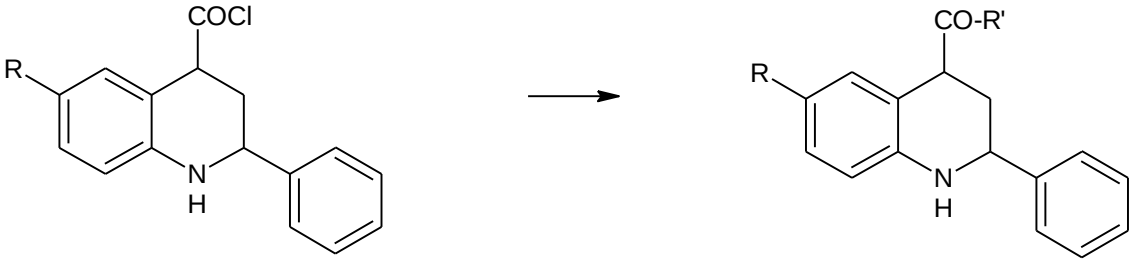
Step 1:



Step 2:



Step 3:



Where R=NO_2; R'=Imidazole/Piperidine/2-amino pyridine

4.3.1. Recrystallization:

Ethanol was added to the synthesized compound and heated until it dissolved completely. The clear solution thus obtained was filtered immediately and set aside for cooling. On cooling crystals gradually appeared.

4.3.2. Methods for Identification:

Melting Point:

The melting points of the compounds were determined by capillary tube method. The temperature at which the synthesized compounds started losing its crystallinity were found and are presented uncorrected.

Thin Layer Chromatography:

Precoated Aluminium TLC-GF binder was used. Solution of the reactant and products in ethanol was prepared. Various mobile phases were tried out of which methanol and chloroform were found to be suitable.

Stationary Phase: Precoated Silica gel GF plates

Mobile phase: Methanol and Chloroform (9:1) as developing solvent system for synthesized compounds

Location of Spots: UV chamber

A single principle spot and absence of secondary spot and spot for parent compounds confirmed the purity of the compounds.

4.3.3. CHARACTERIZATION: [34, 35, 36]

- The compounds were found to have sharp melting points. The melting points are uncorrected.

- The synthesized molecules were subjected to TLC technique and found to be pure. Only a single spot was noticed for the synthesized compound.

- The synthesized molecules were characterized by IR Spectroscopy using ABB Spectrophotometer in the range of 4000 to 400cm^{-1}.

- Proton NMR spectra were recorded using solvent deuterated methanol on BRUKER advance III 500 NMR spectrometer. Chemical shifts are reported in parts per million (ppm).

- The MASS spectra were recorded on JEOL GC Mate II Mass Spectrophotometer operating as direct probe using Electron Ionization (EI) technique.

Step I:

	Reactants				Product (A1-A5)			
	Substituted Aniline	Aldehyde	Pyruvic acid/ Lactic acid	Reaction process	Comp.	Mol. Formula	Mol. Wt.	M.P (°C)
Mole	0.248	0.236	0.25	Reflux 3 hrs	A1	$C_{16}H_{14}N_2O_4$	298.29	282
Volume	23ml	24ml	22ml		A2	$C_{16}H_{10}ClNO_2$	283.70	324
					A3	$C_{18}H_{19}N_3O_4$	341.36	276
					A4	$C_{18}H_{19}N_3O_4$	341.36	276
					A5	$C_{16}H_{10}ClNO_2$	283.70	324

Step II:

	Reactants			Product (B1-B5)			
	A1-A5	Thionyl chloride	Reaction process	Comp.	Mol. Formula	Mol. Wt.	M.P (°C)
Mole	0.01	15ml	Reflux 30 min	B1	$C_{16}H_{13}Cl_2N_2O_3$	316.73	276
Amount	2.83gm			B2	$C_{16}H_9Cl_2NO$	302.15	296
				B3	$C_{18}H_{18}ClN_3O_3$	359.80	312
				B4	$C_{18}H_{18}ClN_3O_3$	359.80	312
				B5	$C_{16}H_9Cl_2NO$	302.15	296

Comp. – Compound, Mol. Formula- Molecular Formula, Mol. Wt.-Molecular weight, M.P-Melting Point.

Step III:

	Reactants				Product (C1-C5)			
	B1-B5	Different Amines	Reaction medium	Reaction process	Comp.	Mol. Formula	Mol. Wt.	M.P (°C)
Mole	0.01	0.03	Ethanol (25ml)	Reflux 5 hrs	C1	$C_{21}H_{18}N_4O_3$	374.39	76
Weight	3.02gm	1)2.82gm 2)2.04gm 3)2.55gm 4)2.04gm 5)2.55gm			C2	$C_{19}H_{12}ClN_3O$	333.77	90
					C3	$C_{23}H_{28}N_4O_3$	408.49	212
					C4	$C_{21}H_{21}N_5O_3$	391.42	214
					C5	$C_{21}H_{19}ClN_2O$	350.84	132

Comp. – Compound

Mol. Formula- Molecular Formula

Mol. Wt.-Molecular weight

M.P- Melting Point

4.4.*PHARMACOLOGICAL EVALUATION:*

4.4.1. *Acute toxicity:* [37]

Acute toxicity is the toxicity produced by the compounds when it is administered in one or more doses during a period not exceeding 24 hours.

Acute toxicity studies in animals are usually necessary for any compounds intended for human use. The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase I human studies, and provide information relevant to acute overdosing in humans.

This acute toxicity study was designed as per the OECD Guidelines for Testing of Chemicals, Acute Oral Toxicity (Acute Toxic Class Method), Guideline 423.

Principle of the test:

Acute toxicity testing determines the toxicity of a chemical or drug substances after single administration.

Today, acute toxicity testing focuses on levels on acute tolerance, nature of acute toxicity symptoms in the sub-lethal range, and dose levels which cause mortality in two animals i.e. quality has replaced quantity.

It is the principle of the test that based on a stepwise procedure with the use of a minimum number of animals per step. The substance was administered orally to a group of experimental animals at one of the defined doses. The substance was tested using a stepwise procedure, each step using five animals of a single sex (normally females). Absence or

presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.

- no further testing is needed.
- dosing of three additional animals, with the same dose.
- dosing of three additional animals at the next higher or the next lower dose level.

Description of the method:

Experimental animals:

Healthy adult female Albino mice (5 nos.) were used in research. Females were weighing between 20-25mg, for all the four animals food, but not water withheld overnight prior to dosing.

Housing and lighting conditions:

The temperature in the experimental animal room should be 22ºC (±3ºC). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but
the number of animals per cage must not interfere with clear observations of each animal.

PROCEDURE:

Administration of doses:

The test substance is administered in a single dose by 19 gauge using an oral tube.

Testing procedures:

The test compounds should be administered to animal to identify doses causing no adverse effects and doses causing major (life-threatening) toxicity. The use of vehicle control groups should be considered. For compounds with low toxicity, maximum feasible dose should be administered.

Studies should be conducted in at least two mammalian species, including a non-rodent species when reasonable. The objectives of acute studies can usually be achieved in rodents using small groups of animals (for instance, three to five rodents per sex per dose). Where non-rodent species are appropriate for investigation, use of fewer animals may be considered. Any data providing information on acute effects in non-rodent species, including preliminary dose-range finding data for repeat-dose toxicity studies, may be acceptable.

OBSERVATION:

The animals were observed individually after dosing once during the first 30 minutes, periodically for the first 24 hours, with special attention given during the first 4 hours & daily there-after, for a total of 14 days. The following clinical observation were made and recorded.

- Toxic Signs:

All mice were observed for any toxic signs.

All the rats were observed for any pre-terminal deaths.

- Body Weight:

  Individual body weights were recorded for all the animals once in a week.

- Cage Side Observation:

  The faeces colour, faeces consistency, changes in skin & fur, eyes & mucous membrane (nasal) of the animal were observed once in a week.

- Physical examination:

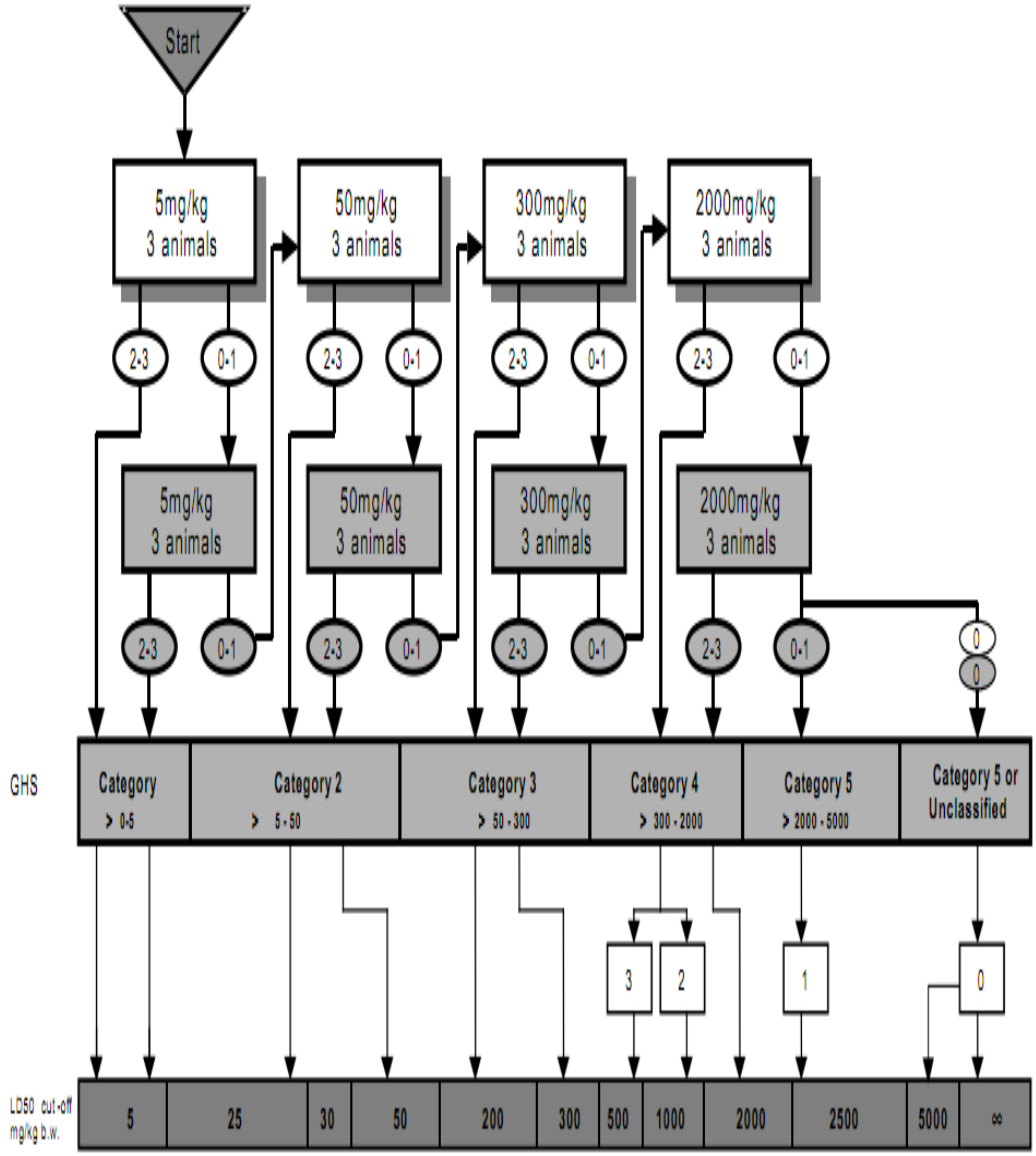
  Physical examination included changes in

  - Salivation, lacrymation, perspiration, piloerection, micturition and defecation.

  - Central nervous system:

    Ptosis, drowsiness, gait, eye prominence, eyelid closure, convulsions, biting, straub's test, motor incoordination, writhing, stereotypy, aggression, righting reflex, pinna reflex, corneal reflex, tremors and convulsions.

ANNEX 2a: TEST PROCEDURE WITH A STARTING DOSE OF 5 MG/KG BODY WEIGHT



4.4.2. *IN VITRO ANTI CANCER ACTIVITY:* [38, 39]

The human colorectal carcinoma cell line (HCT116) was obtained from National Centre for Cell Science (NCCS), Pune, and grown in Dulbeccos Modified Eagles Medium (DMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37^0C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium with 5% FBS to give final density of $1x10^5$ cells/ml. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the extracts and fractions. They were initially dissolved in neat dimethylsulfoxide (DMSO) and further diluted in serum free medium to produce five concentrations. One hundred microlitres per well of each concentration was added to plates to obtain final concentrations of 100, 10, 1.0 and 0.1 µM. The final volume in each well was 200 µl and the plates were incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 48h. The medium containing without samples were served as control. Triplicate was maintained for all concentrations.

MTT assay: [40, 41]

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC50 was determined using GraphPad Prism software.

RESULTS AND DISCUSSION

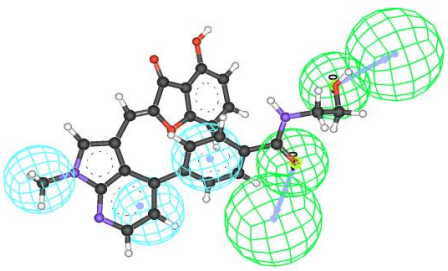
5. RESULTS AND DISCUSSION

Our efforts to synthesize novel anticancer compounds based on drug design lead to the following results and discussed accordingly.

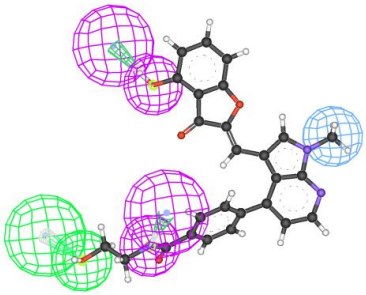
5.1.*Pharmacophore model and validation:*

The main purpose of validating a quantitative pharmacophore model is to determine whether it is capable of identifying active compounds predicting their activities accurately and further performing database searching. Two validation procedures were followed namely, Test set prediction method and Cat-scramble method. The Cat-scramble validation procedure is a cross-validation based on Fischer's randomization test. The goal of this type of validation is to check whether there is a strong correlation between the chemical structures and the biological activity. This is done by randomizing the activity data associated with the training set compounds, generating pharmacophore hypotheses using the same features and parameters to develop the original pharmacophore model.

17 run Active:

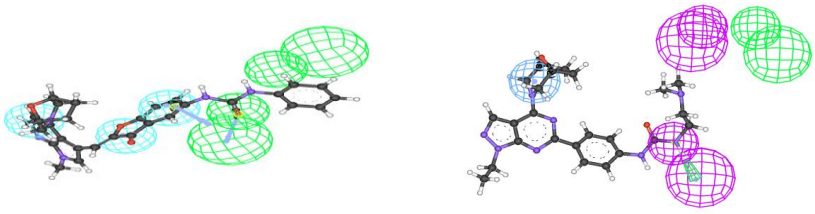


15 run Active:



17 run Inactive:

15 run Inactive:



Pharmacophore: Pharmacophore distance:

Fig. 9 Pharmacophore modeling

Table 4:

Statistical parameters from screening of test set molecules:

S.No.	Parameter	mTOR
1	Total compounds in database (D)	297
2	Total Number of actives in database (A)	170
3	Total Hits (H_t)	176
4	Active Hits (H_a)	162
5	% Yield of Actives [(H_a/ H_t)*100]	92.05
6	% Ratio of actives in the Hit list [(H_a/A)*100]	95.29
7	Enrichment factor or Enhancement (E) [(H_a * D)/(H_t*A)]	1.61
8	False Negatives [A-H_a]	8
9	False Positives [H_t- H_a]	14
10	GH score (Goodness of Hit list)	0.83

[(H_a/4H_tA) (3A+ H_t))* (1-((H_t- H_a)/(D-A))]; GH score of 0.7-0.8 indicates a very good model.

The best common feature pharmacophore model hypo 1 (from outhypo 17) and hypo 3 (from outhypo 15) indicated the importance of H-bond acceptor (HBA), H-bond donor (HBD), Hydrophobic (HY), and ring aromatic (RA) features, which were further confirmed in the quantitative models. 3D QSAR pharmacophore Generation module/Discovery studio (DS) was used to construct pharmacophore model using the above mentioned features. It produced ten top-scored hypotheses based on the activity values of the training set molecules. The best ten hypotheses contain only HBA, HBD, RA and Hydrophobic features. Hypo 1 consist of 2 HBA, 3 HY and Hypo 3 consist of HBA, HBD, RA which establishes the highest cost difference, lower errors, best correlation coefficient, maximum fit value, and lowest root mean square deviation (RMSD) (table 5 & 6). The fixed and the null costs values are 99.7202(for outhypo 17), 98.8074(for outhypo 15) and 140.601 respectively. Fixed total cost was dependent on summation of the cost components: weight cost, error cost, and configuration cost. Two key values were used for cost analysis: one is the difference between the fixed and null costs and another one is the difference between null and total costs (cost difference). The fixed cost represents a cost of the theoretical ideal hypothesis, which could absolutely predict the activity of compounds in the training set with lowest deviation, while null cost represented the cost of hypothesis with no features that estimates every activity to be the average activity. The difference between these two costs should be ≥70 bits to show the over 90% statistical significance of the model. The cost difference should be greater than 60 bits to represent a true correlation data.

Pharmacophore Models generated by HypoGen:

Table 5:

Outhypo 17:

Hypo.No.	**Total cost**	**Fixed cost**	**Cost difference**	**RMSD**	**Correlation**	**Features**	**Max. fit**
1	117.729	99.7202	22.872	1.2249	0.836	HBA,HBA,HY,HY,HY	10.1229
2	117.824	99.7202	22.777	1.2217	0.837	HBA,HBA,HY,HY,HY	10.9338
3	117.875	99.7202	22.726	1.2296	0.834	HBA,HBA,HY,HY,HY	9.7913
4	118.19	99.7202	22.411	1.2383	0.832	HBA,HBA,HY,HY,HY	9.443
5	118.755	99.7202	21.846	1.2593	0.825	HBA,HBD,HBD,HY	8.0423
6	118.76	99.7202	21.841	1.2387	0.833	HBA,HBA,HY,HY,HY	11.6888
7	118.946	99.7202	21.655	1.2654	0.824	HBA,HBD,HBD,HY	8.1714
8	120.155	99.7202	20.446	1.3005	0.813	HBA,HBA,HBA,HY	8.6328
9	120.875	99.7202	19.726	1.3216	0.806	HBA,HBD,HBD,HY	8.7544
10	120.949	99.7202	19.652	1.3281	0.804	HBA,HBA,HY,HY,HY	9.4722

Null Hypothesis:

Total cost – 140.601; RMS – 2.23373; Error – 140.601

Pharmacophore Models generated by HypoGen:

Table 6:

Outhypo 15:

Hypo.No.	Total cost	Fixed cost	Cost difference	RMSD	Correlation	Features	Max. fit
1	123.316	98.8074	17.285	1.4290	0.7685	HBA,HBD,HBD,HYALI	7.9201
2	123.765	98.8074	16.836	1.3811	0.7932	HBD,HYALI,RA,RA	10.4565
3	123.767	98.8074	16.834	1.3173	0.8263	HBA,HBD,RA	8.6055
4	124.173	98.8074	16.428	1.4517	0.7601	HBA,HBD,HBD,HYALI	8.4634
5	124.504	98.8074	16.097	1.4289	0.7723	HBD,HBD,HYALI,RA	9.8660
6	124.735	98.8074	15.866	1.4691	0.7533	HBA,HBA,HBD,HYALI	8.2707
7	124.811	98.8074	15.79	1.4425	0.7666	HBD,HBD,HYALI,RA	9.7348
8	124.845	98.8074	15.756	1.4266	0.7750	HBD,HYALI,RA,RA	10.1712
9	124.846	98.8074	15.755	1.4065	0.7856	HBD,HYALI,RA,RA	10.5895
10	125.056	98.8074	15.545	1.4697	0.7532	HBD,HBD,HYALI,RA	8.9747

Null Hypothesis:

Total cost – 140.601; RMS – 2.2337; Error – 140.601

Experimental and Predicted IC50 data of 24 training set molecules:

Table 7:

Outhypo 15(Hypo 3)

Compound No.	IC_{50}		ERROR	FIT VALUE	ACTIVITY SCALE		MAPPED FEATURES		
	EXPERIMENTAL	PREDICTED			Experimental	predicted	HBA	HBD	RA
1	0.0016	0.047	29	7.99	+++	+++	18	30	16
2	0.29	4.2	15	6.04	+++	++	11	17	16
3	0.35	0.55	1.6	6.92	+++	+++	19	14	16
4	0.42	0.34	-1.2	7.13	+++	+++	20	11	10
5	0.46	3.1	6.7	6.17	+++	++	21	23	16
6	0.63	3.3	5.3	6.14	+++	++	17	13	12
7	0.75	3.3	4.4	6.15	+++	++	20	15	13
8	0.9	1.1	1.2	6.62	+++	++	13	17	16
9	1.2	0.17	-7.1	7.43	++	+++	-17	-20	-18
10	1.6	10	6.5	5.64	++	++	-19	-13	-12
11	2.6	1.5	-1.7	6.48	++	++	19	15	13
12	2.9	1.4	-2.1	6.52	++	++	17	14	12
13	3.9	2.9	-1.3	6.19	++	++	-14	-11	-1
14	5.6	1.6	-3.5	6.46	++	++	16	27	7
15	7	3.2	-2.2	6.15	++	++	-35	-31	-6

16	9.6	14	1.4	5.53	++	+	17	19	*
17	13	15	1.1	5.49	+	+	-12	-17	-1
18	18	14	-1.3	5.53	+	+	-15	-17	-1
19	26	18	-1.4	5.40	+	+	*	-16	-1
20	34	8.6	-3.9	5.73	+	++	-17	-14	-1
21	51	15	-3.4	5.48	+	+	12	20	15
22	71	11	-6.2	5.60	+	+	-24	-17	-6
23	94	24	-3.8	5.27	+	+	*	10	13
24	140	22	-6.7	5.32	+	+	16	20	*

Experimental and Predicted IC50 data of 24 training set molecules:

Table 8:

Outhypo 17 (Hypo 1)

COMPOUND NO.	IC_{50}		ERROR	FIT VALUE	ACTIVITY SCALE		MAPPED FEATURES				
	EXPERIMENTAL	PREDICTED			Experimental	Predicted	HBA	HBA	HY	HY	HY
1	0.0016	0.0033	2.1	9.85	+++	+++	30	19	21	29	25
2	0.29	2.7	9.1	6.94	+++	++	-9	-6	-23	*	-33
3	0.35	1.1	3.1	7.34	+++	++	-4	-8	-30	*	-28
4	0.42	2.7	6.5	6.93	+++	++	2	4	26	*	23
5	0.46	1.8	3.8	7.12	+++	++	11	3	14	36	*
6	0.63	0.98	1.6	7.38	+++	+++	-7	-6	-23	*	-20
7	0.75	1	1.4	7.35	+++	++	7	6	22	*	25
8	0.9	1.2	1.3	7.30	+++	++	-8	-4	-23	*	-35
9	1.2	1.7	1.4	7.13	++	++	9	4	33	*	37
10	1.6	1.1	-1.4	7.32	++	++	-4	-8	-27	*	-29
11	2.6	0.91	-2.8	7.41	++	+++	7	6	30	*	29
12	2.9	2.7	-1.1	6.93	++	++	7	6	23	*	32
13	3.9	3.9	-1	6.78	++	++	-3	-4	20	*	-34
14	5.6	4.5	-1.3	6.72	++	++	16	12	11	31	*
15	7	3.1	-2.2	6.87	++	++	13	35	28	37	*
16	9.6	57	6	5.61	++	+	6	7	28	*	*
17	13	0.75	-17	7.50	+	+++	9	4	22	*	34
18	18	58	3.2	5.60	+	+	-6	-5	-28	*	*

19	26	41	1.5	5.75	+	+	19	*	8	13	26
20	34	36	1	5.82	+	+	*	-16	-18	*	-34
21	51	33	-1.6	5.85	+	+	*	19	22	*	34
22	71	22	-3.3	6.03	+	+	*	*	25	14	27
23	94	3.1	-30	6.88	+	++	-2	-5	-24	*	-11
24	140	26	-5.5	5.95	+	+	*	*	4	28	31

5.2.*Docking studies:*

Molecular docking was executed for accurate docking of ligand into protein active sites. Docking experiments were performed using Glide®. For docking study, initially protein was prepared by removing all water molecules. After the protein preparation, the active sites in the protein have to be identified. The active site of the protein was represented as binding site; it's a set of points on a grid that lie in a cavity. The binding site for the protein is defined by the volume occupied by the known ligand pose already in an active site. During docking process, Glide initially performs a complex systematic search of conformation, orientation and position of a compound in a defined binding site and eliminated unwanted poses using scoring and energy optimization. Finally the conformations were refined via a Monte Carlo Sampling. Docking poses were energy minimized using OPLS-2001 force field. The best pose was selected based on Glide score and the favorable interactions formed between the compound and amino acid residues of the mTOR active site. All the ligands in the complex structures showed the hydrogen bond interactions with Asp 64, Asp 177, Lys 7, Val 60, Gly 64, and Asp 15 (fig.10). This clearly indicates that these hydrogen bonded amino acids play a crucial role in mTOR inhibition

activity. During the docking process top 10 conformations was generated for each ligand based on dock score value after the energy minimization process.

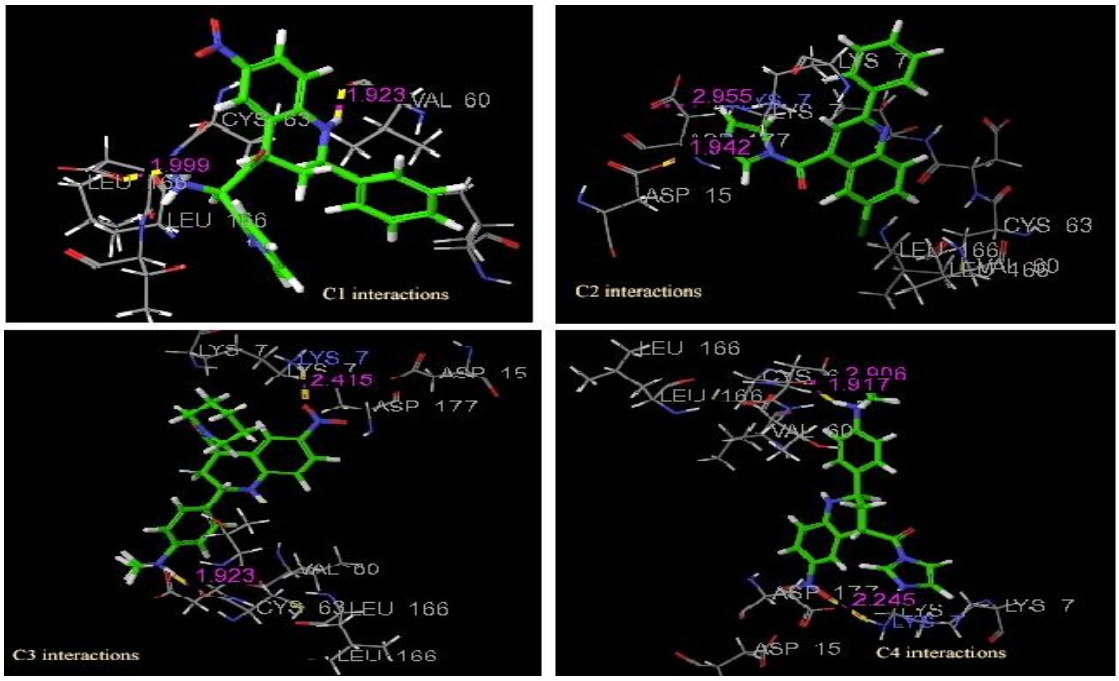
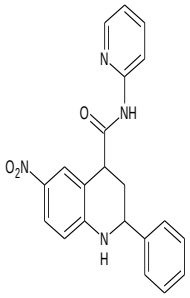
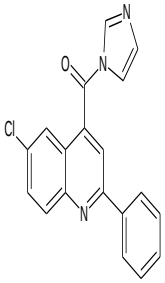
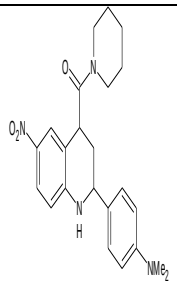
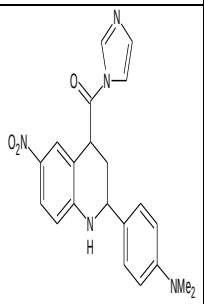
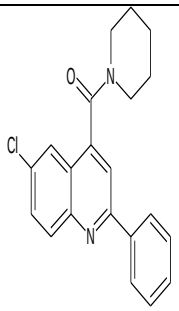


Fig. 10 Interactions

5.3.*Synthesis and characterization:*

Table 9:

Synthesis and identification of Quinoline and Tetrahydroquinoline derivatives:

Compound no.	C-1	C-2	C-3	C-4	C-5
Product					
Recrystallization	Ethanol	Ethanol	Ethanol	Ethanol	Ethanol
Yield (%)	76	78	75	80	77
Melting Point	76^0C	90^0C	212^0C	214^0C	132^0C
TLC Stationary Phase	Precoated silica gel GF plates	Precoated silica gel GF plates	Precoated silica gel GF plates	Precoated silica gel GF plates	Precoated silica gel GF plates

Mobile Phase	Methanol/ Chloroform	Methanol/ Chloroform	Methanol/ Chloroform	Methanol/ Chloroform	Methanol/ Chloroform
Location of spots	UV Chamber	UV Chamber	UV Chamber	UV Chamber	UV Chamber
IR spectroscopy (cm^{-1})	1304(C-N) 1474(Ar C=C) 1597(C-NO_2) 1636(C=O) 2962(Ar C-H) 3479(N-H)	756(C-Cl) 1311(Ar C-N) 1628(Ar C=C) 1674(C=O of Amide) 2932(Ar C-H)	1319(C-N) 1373 cm^{-1} ($N(CH_3)_2$) 1458(Ar C=C) 1551(C-NO_2) 1651(C=O) 3070(Ar C-H)	1319(C-N) 1373 cm^{-1} ($N(CH_3)_2$) 1458(Ar C=C) 1551(C-NO_2) 1643(C=O) 3063(Ar C-H)	764(C-Cl) 1311(Ar C-N) 1497(Ar C=C) 1674(C=O of Amide) 2978(Ar C-H)
NMR spectroscopy	1.6 (4 H) 6.1(4H,Ar) 6.6(4H,Ar) 7.2(1,NH) 8.1(4H, Het.Ar) 9.9(1H,NH)	6.6(3H,Ar) 7.5(5H,Ar) 7.9(3H, Het.Ar) 8.4(1H, Het.Ar)	2.4(6H, $N(CH_3)$) 2.9(4H) 3.2(10H) 7.0(1H,NH) 7.6-9.6 (7H,Ar)	2.4(4H) 3.3(6H, $N(CH_3)$) 6.2-6.9 (7H,Ar) 7.6-7.9(3H, Het.Ar) 9.7(1H,NH)	1.6(10H) 6.1,6.4,6.6 (8H,Ar) 7.9(1H, Het.Ar)
MASS spectrometry	374.41(M^+, 6%) 140.30 (B,100%)	333.38(M^+,12%) 61.77 (B,100%)	408.52(M^+,6%) 61.77 (B,100%)	391.42(M^+,7%) 135.21 (B,100%)	350.88(M^+,10%) 62.57 (B,100%)

IR spectrum of compound 1:

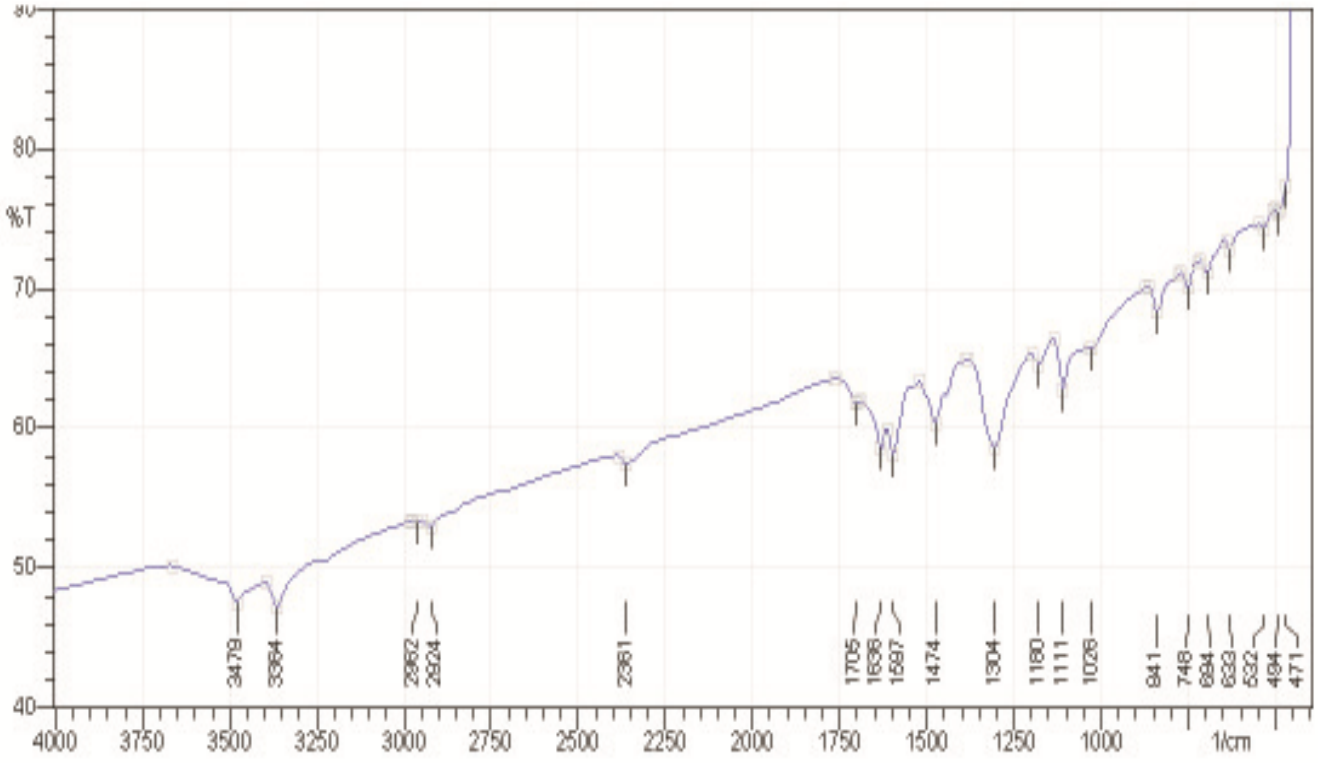


Fig. 11

NMR spectrum of compound 1:

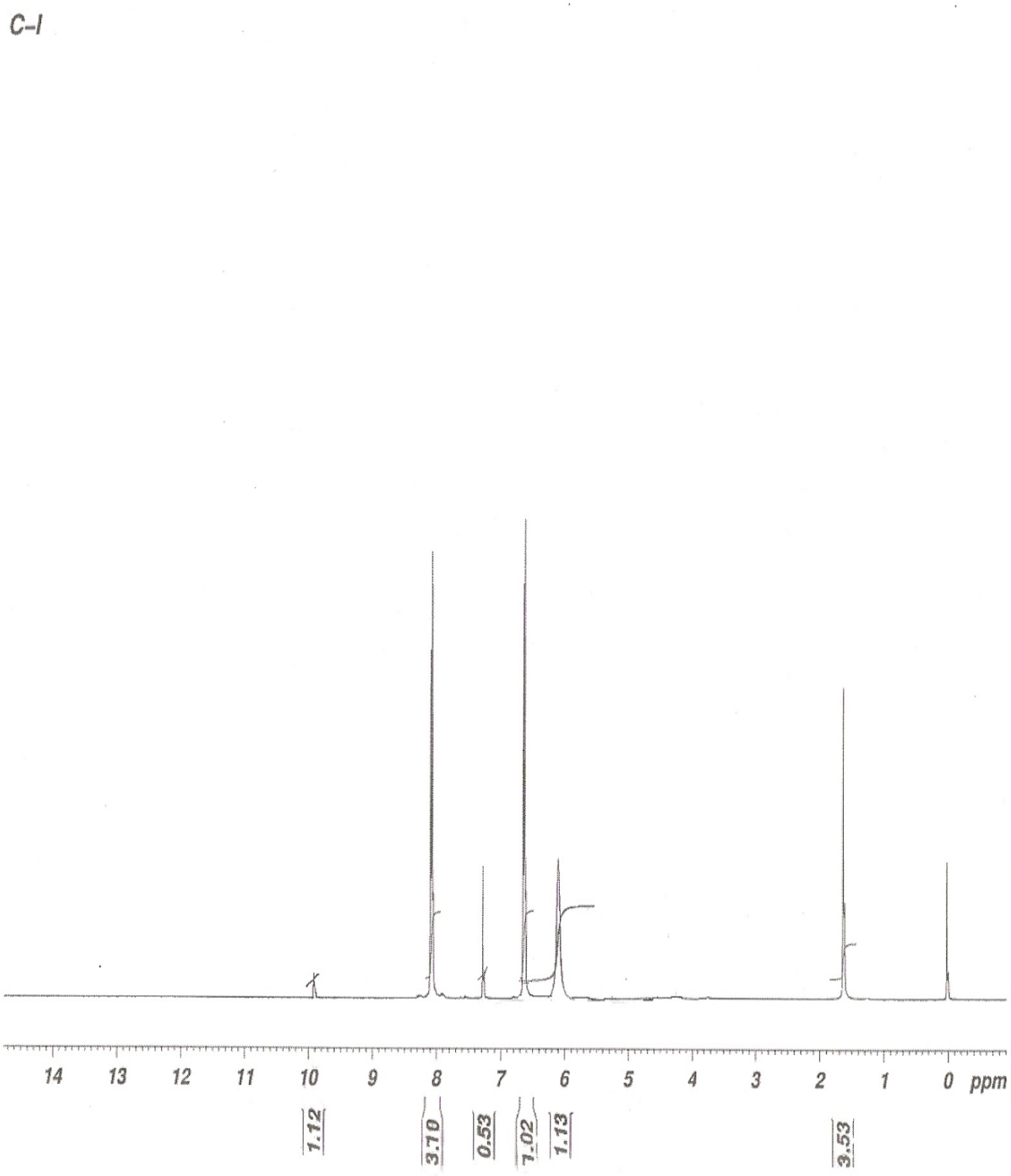


Fig. 12

MASS spectrum of compound 1:

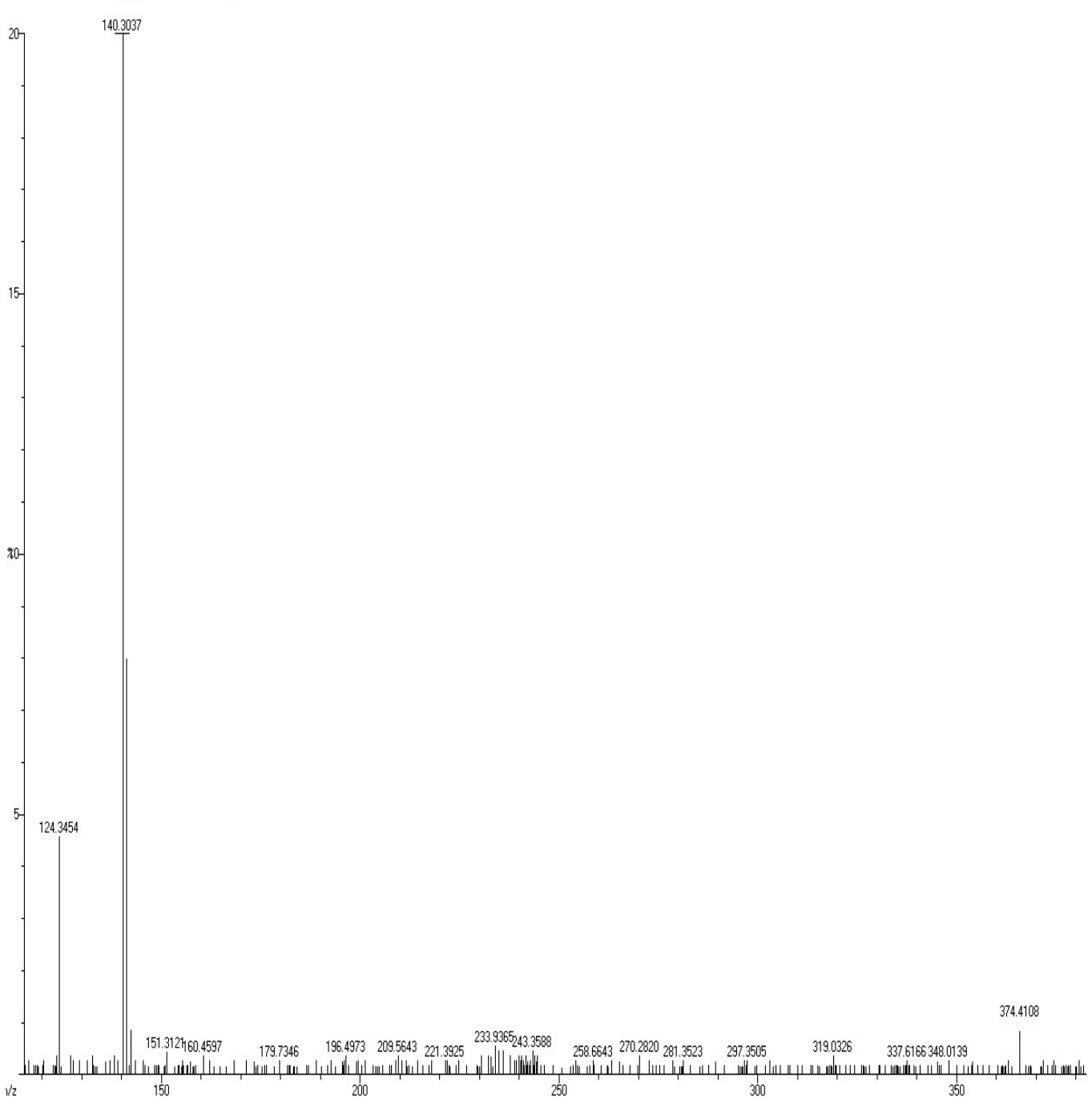


Fig. 13

IR spectrum of compound 2:

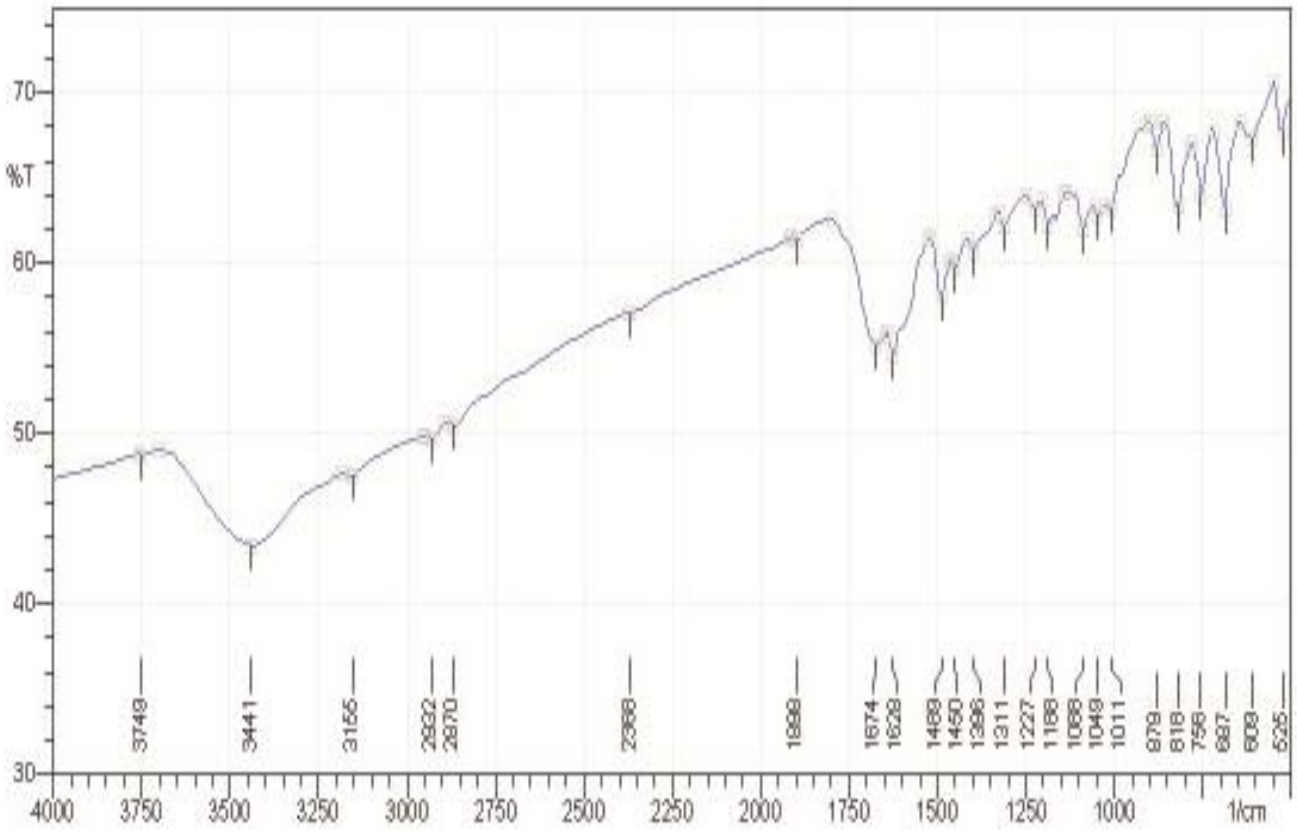


Fig. 14

NMR spectrum of compound 2:

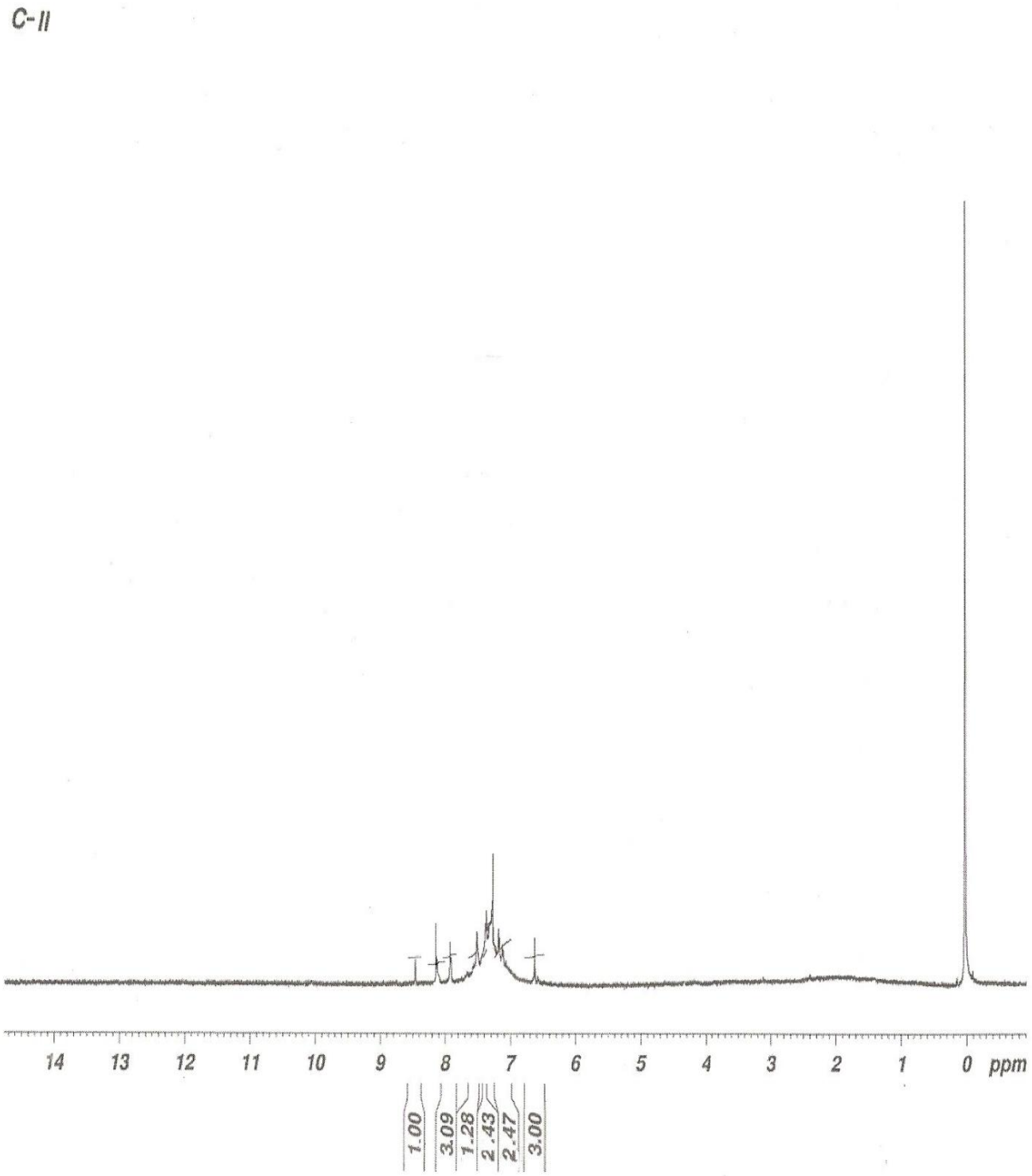


Fig. 15

MASS spectrum of compound 2:

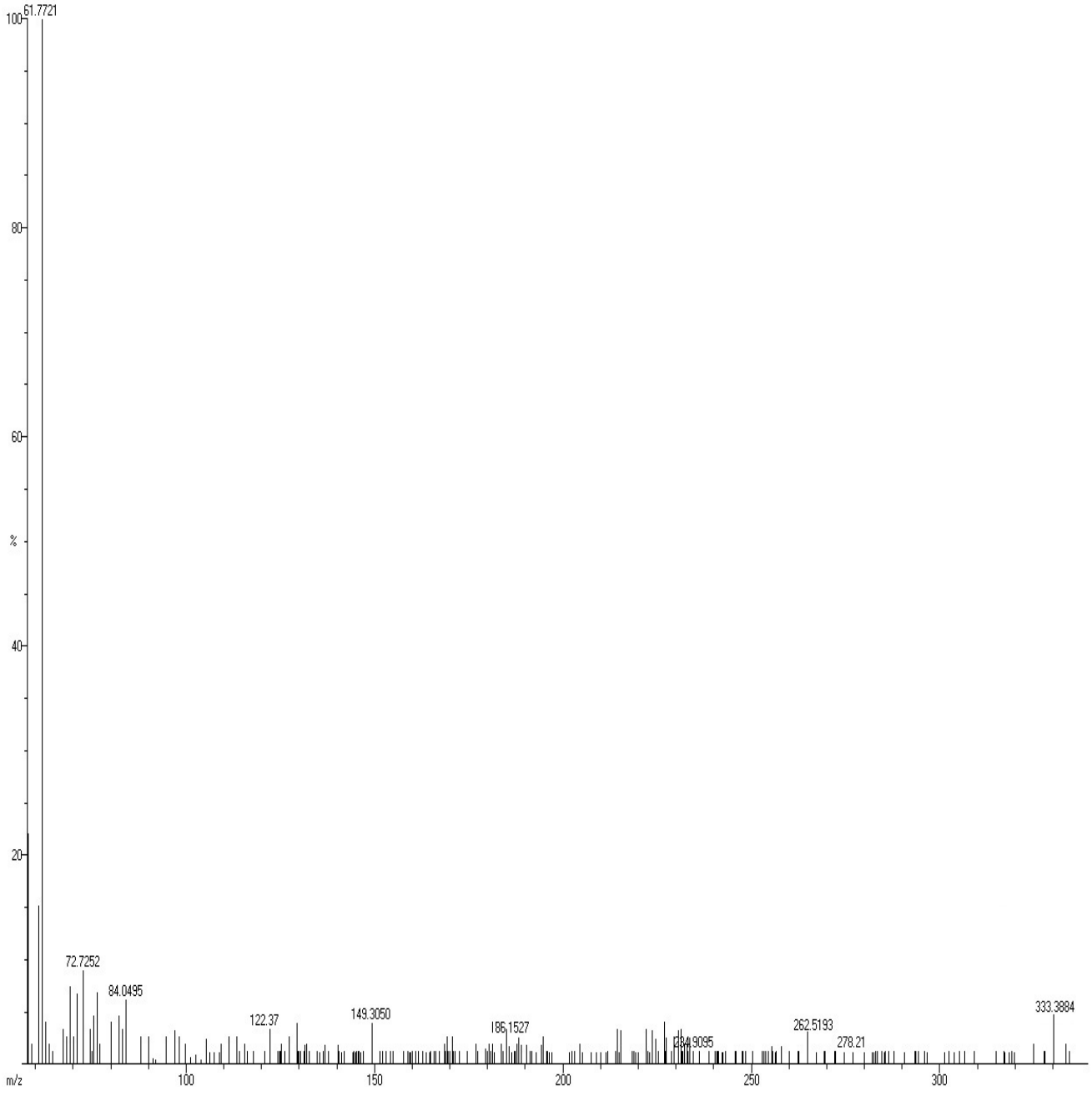


Fig. 16

IR spectrum of compound 3:

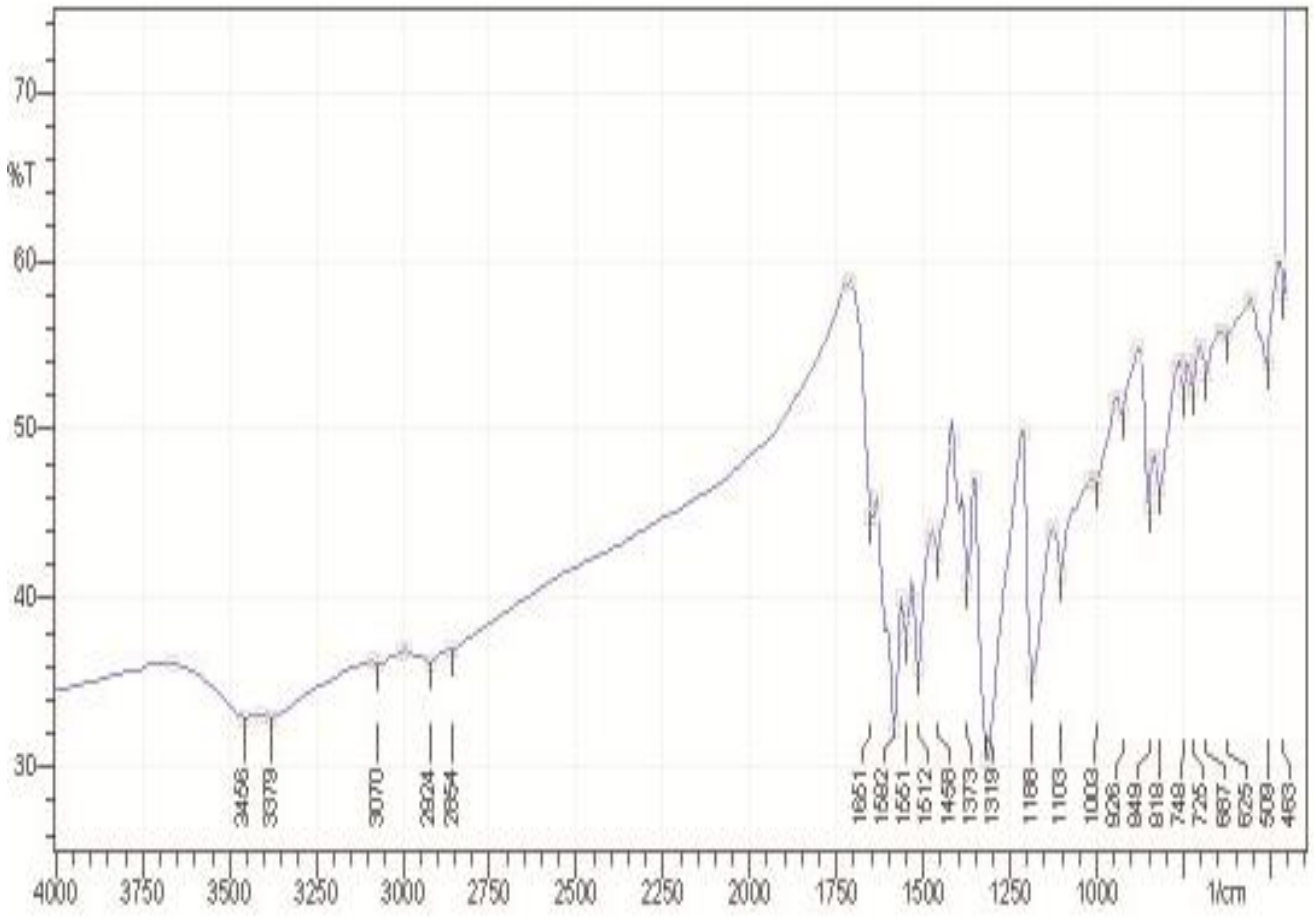


Fig. 17

NMR spectrum of compound 3:

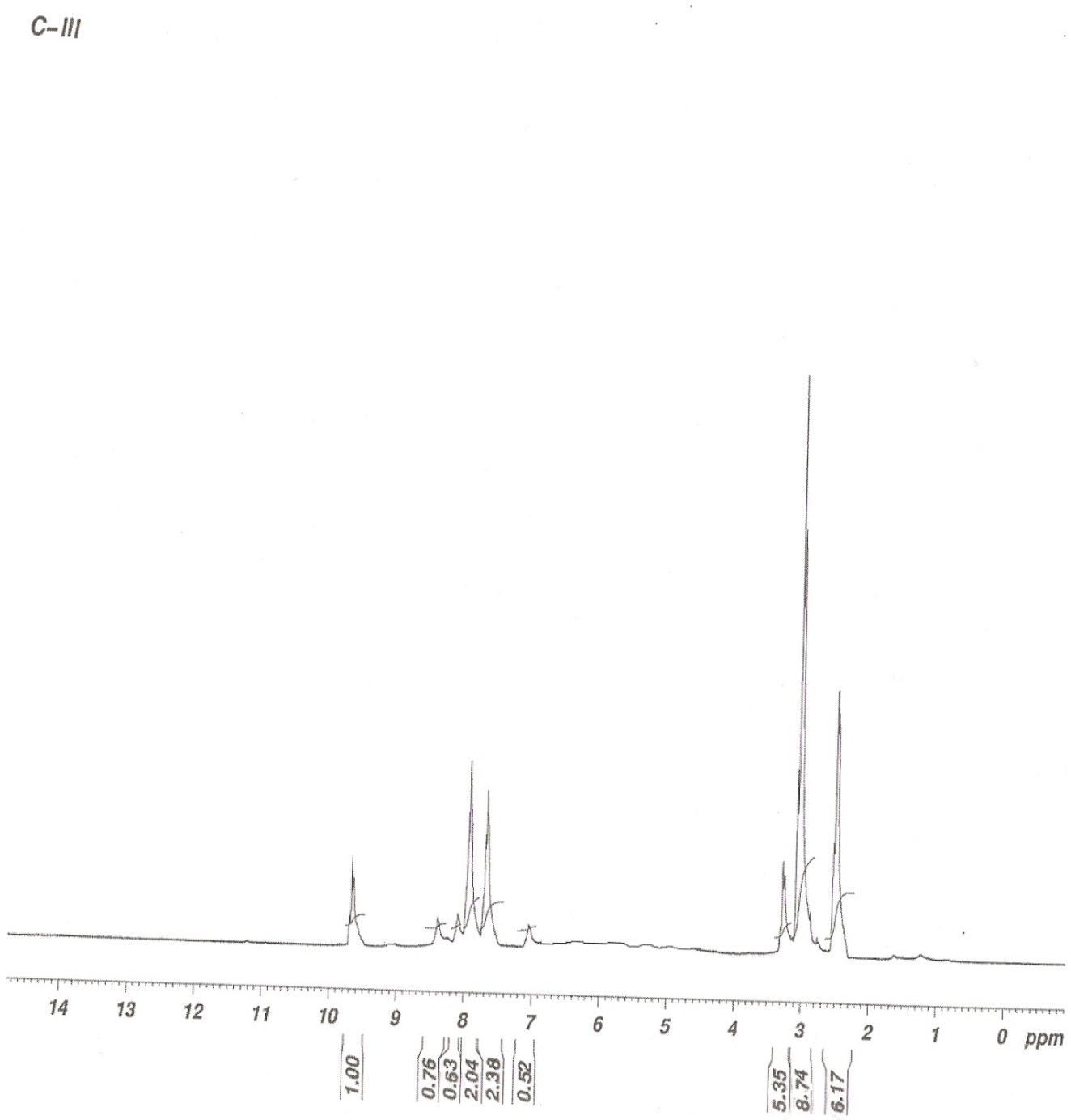


Fig. 18

MASS spectrum of compound 3:

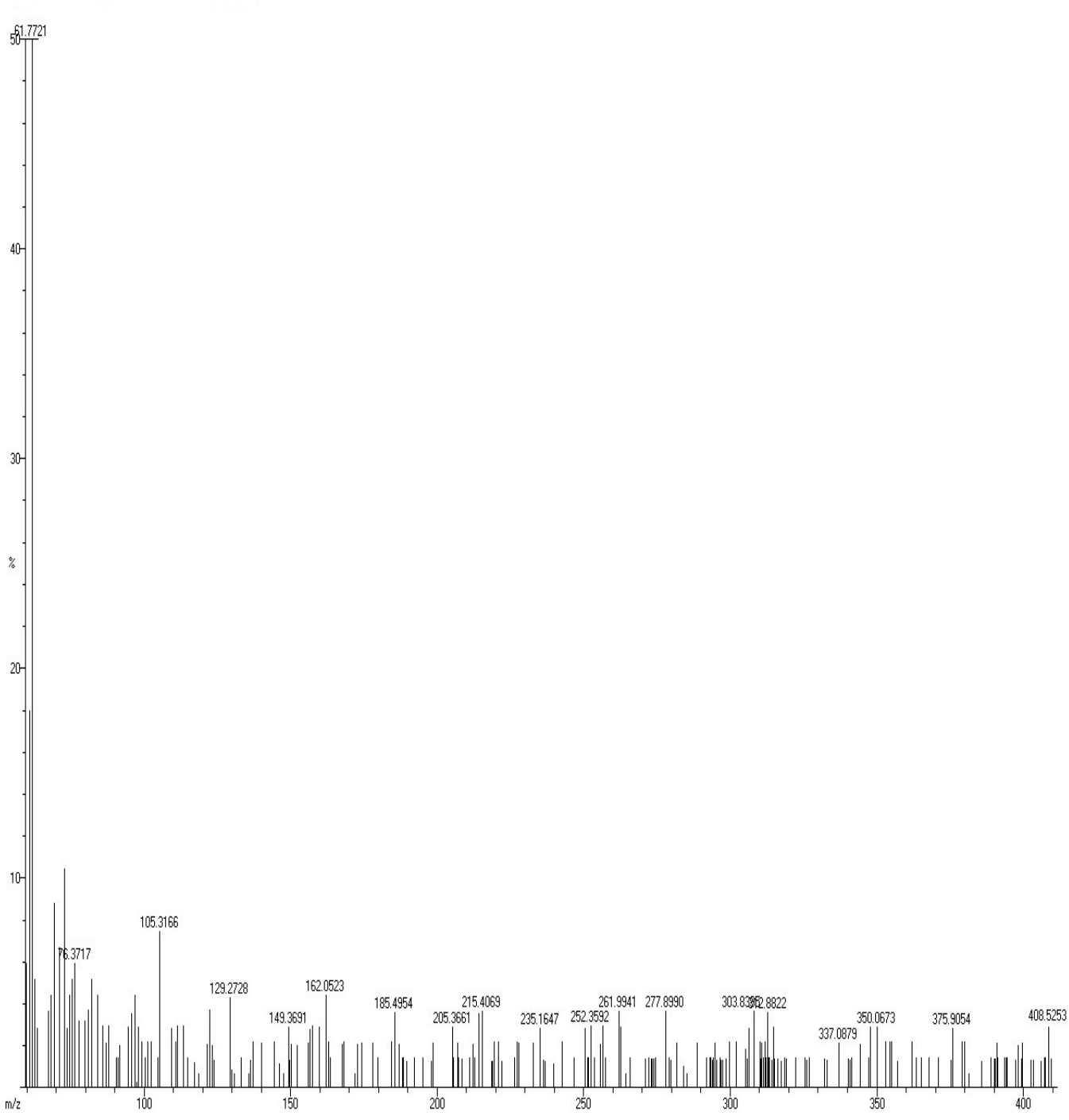


Fig. 19

IR spectrum of compound 4:

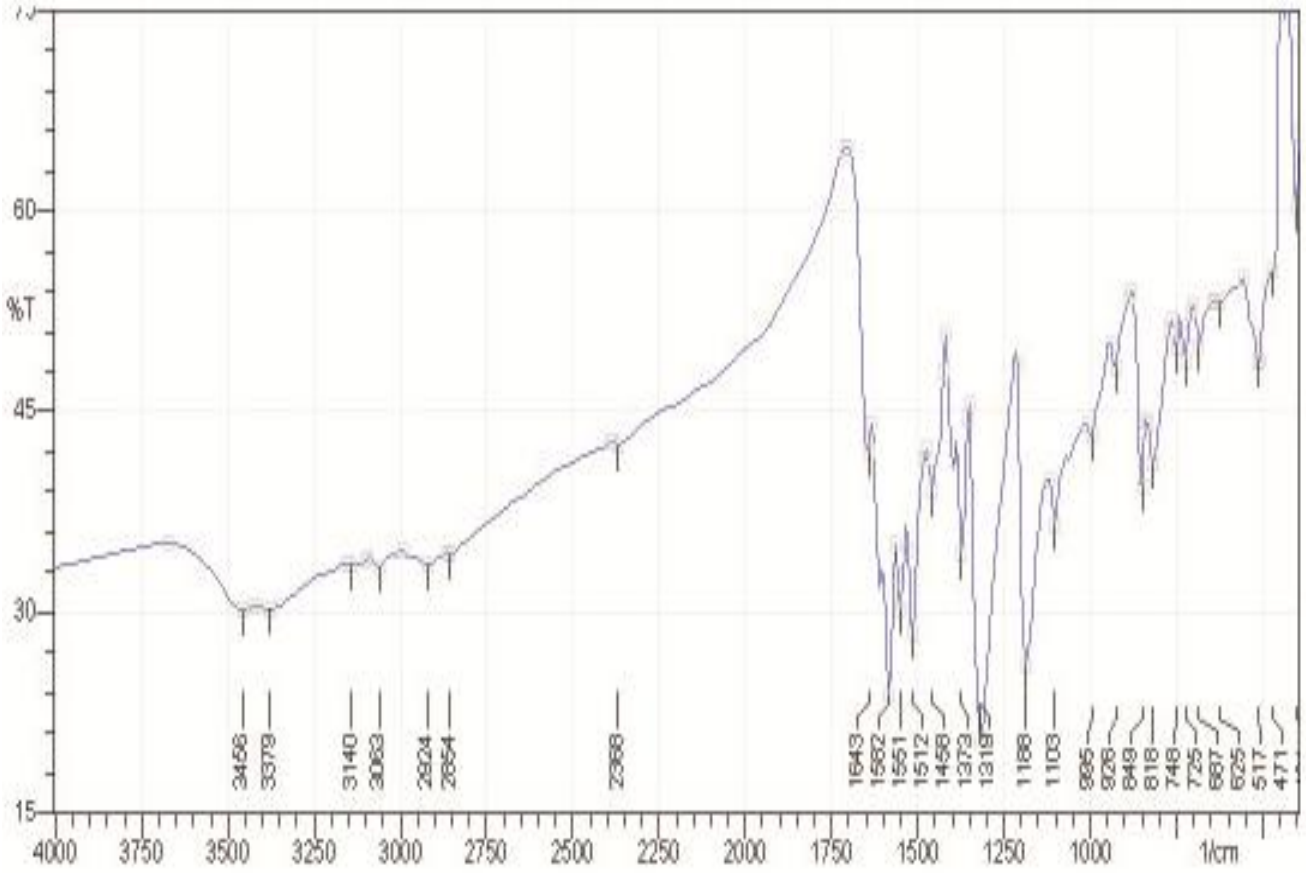


Fig. 20

NMR spectra of compound 4:

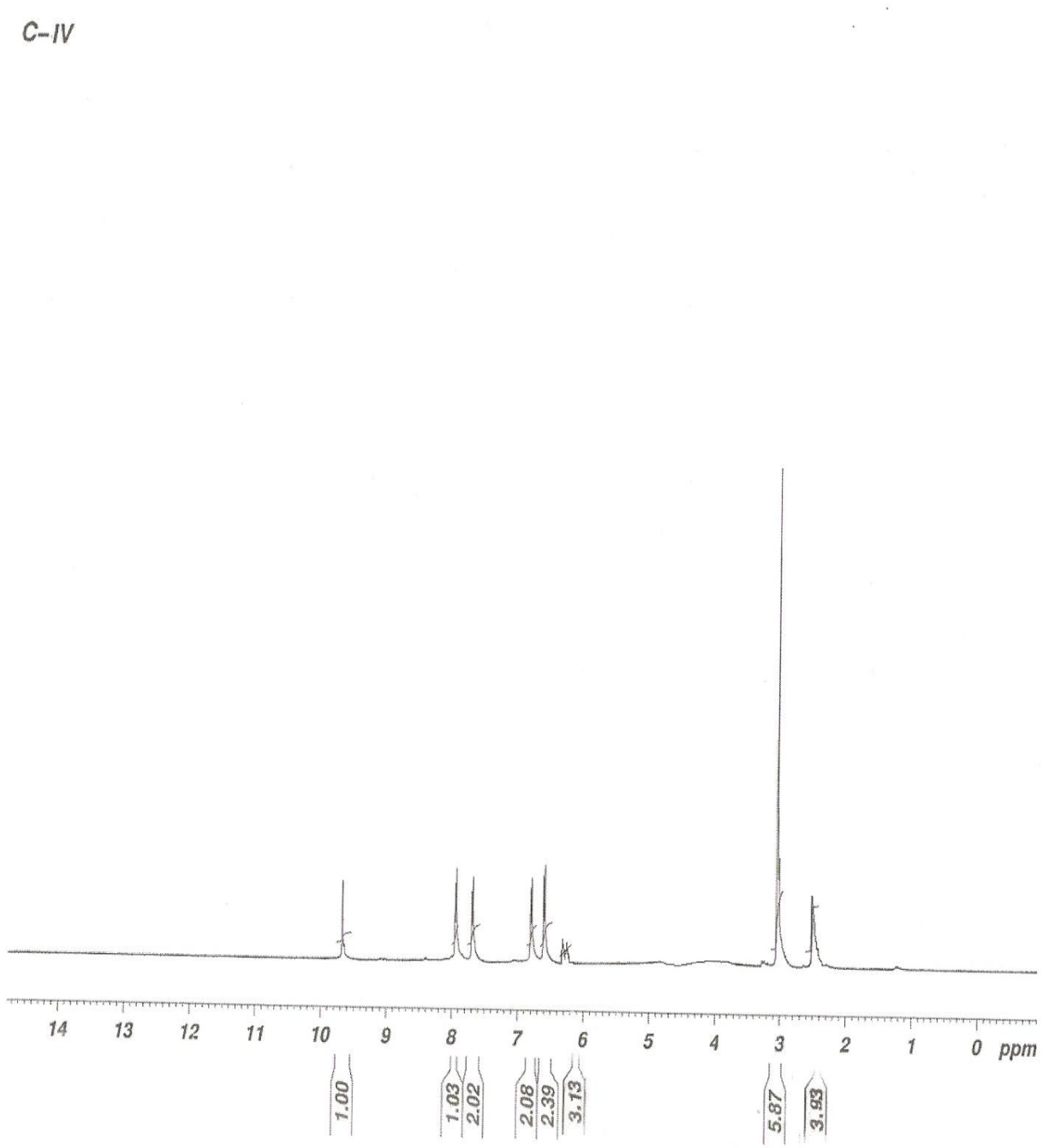


Fig. 21

MASS spectrum of compound 4:

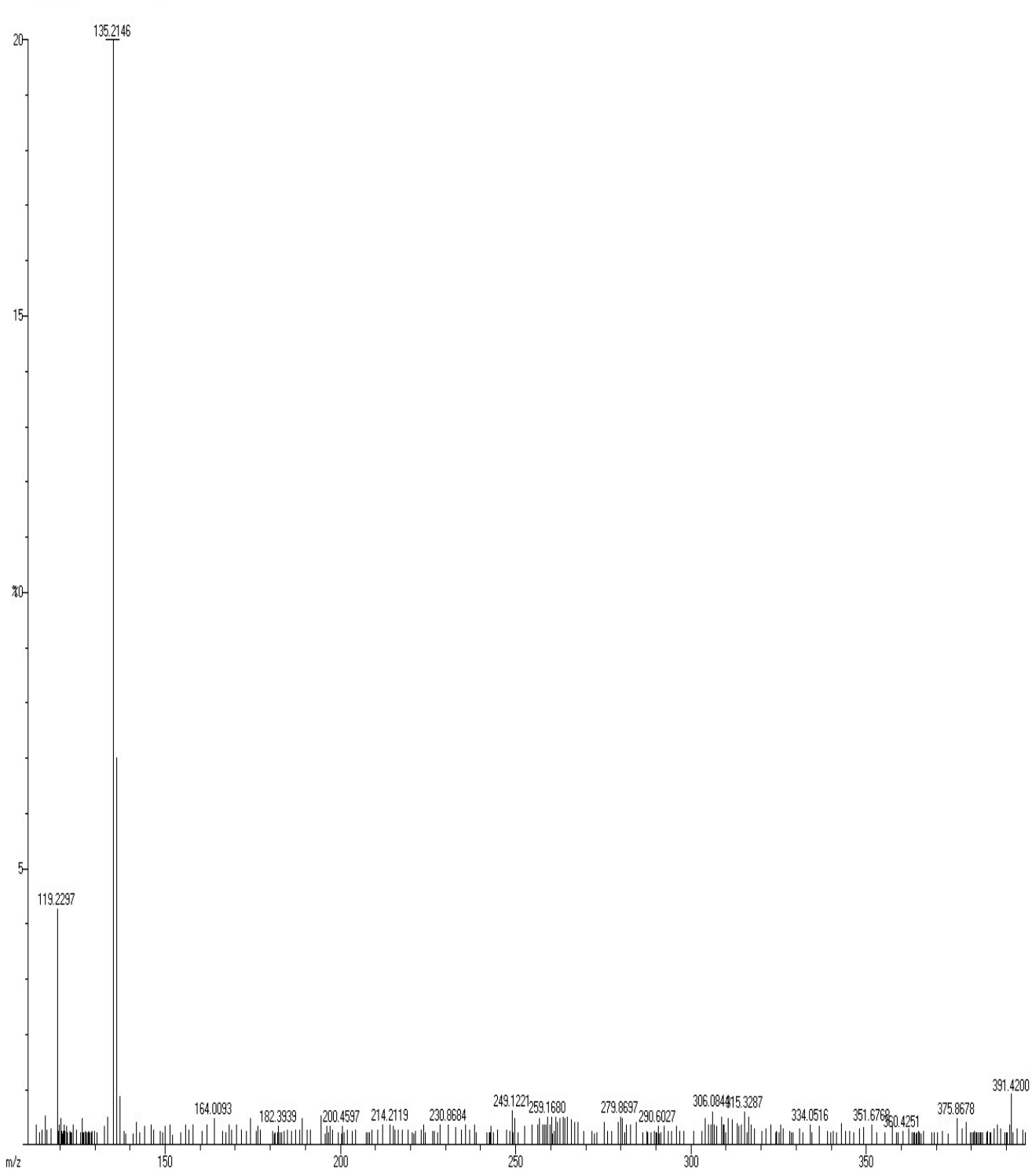


Fig.22

IR spectrum of compound 5:

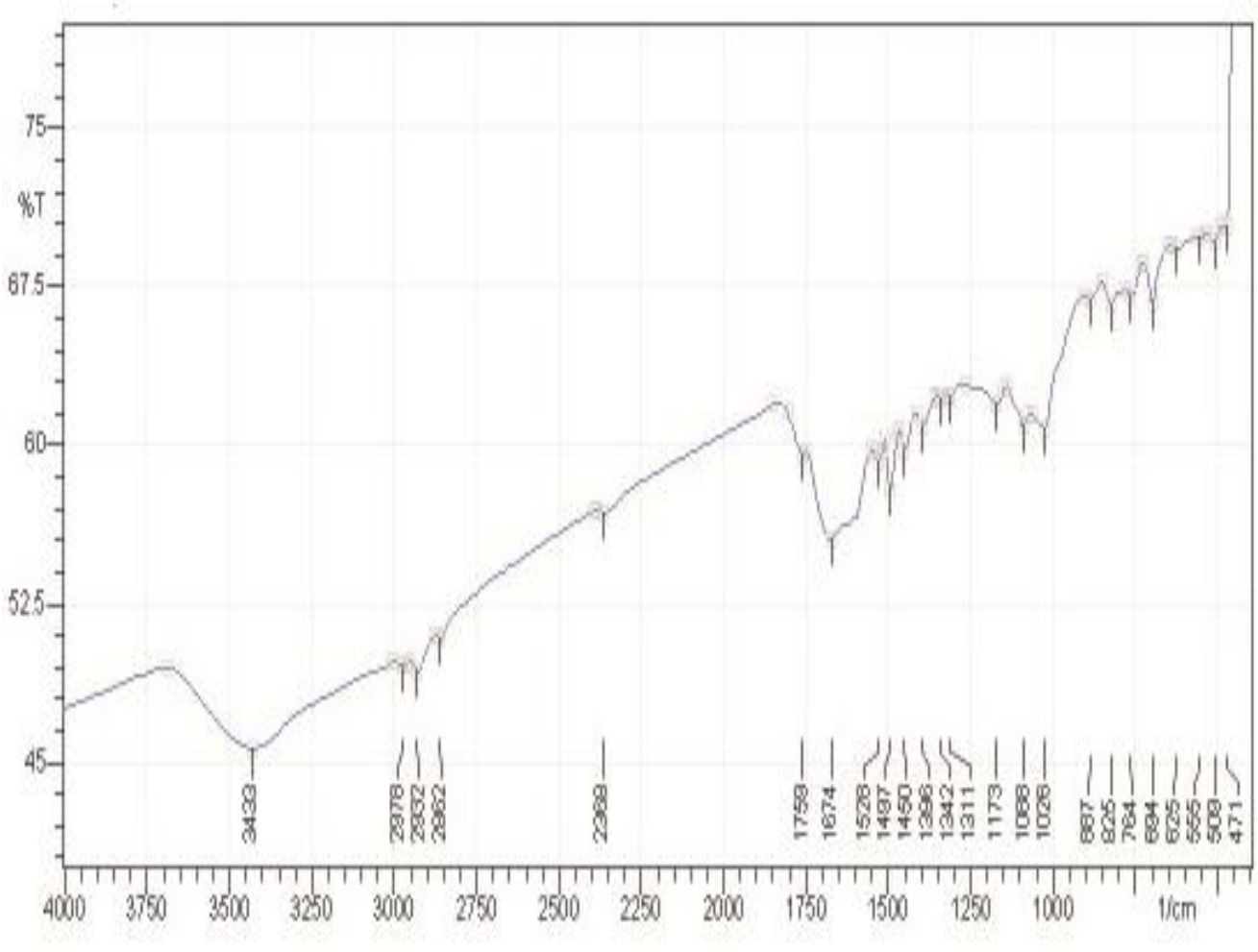


Fig.23

NMR spectrum of compound 5:

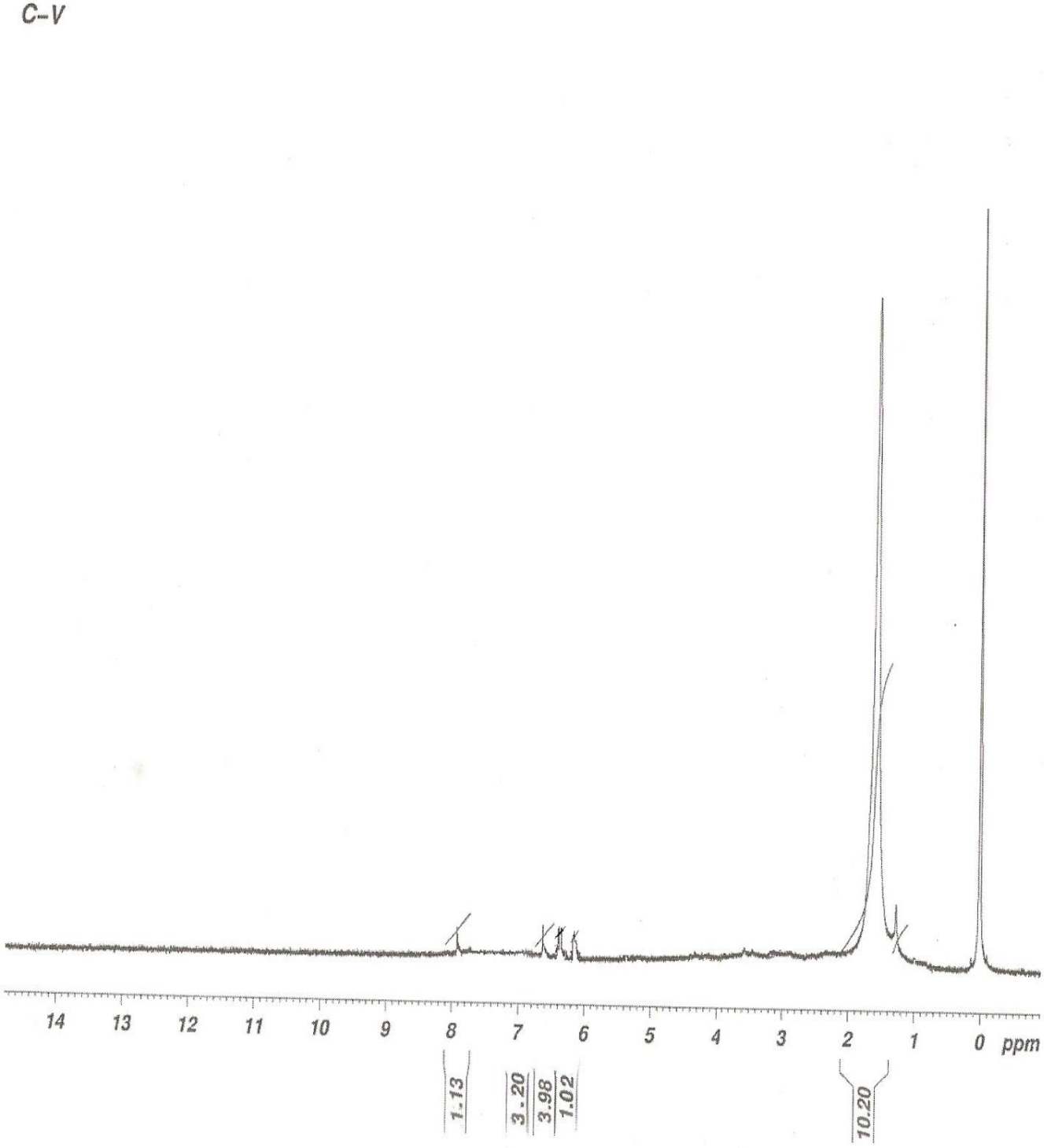


Fig.24

MASS spectrum of compound 5:

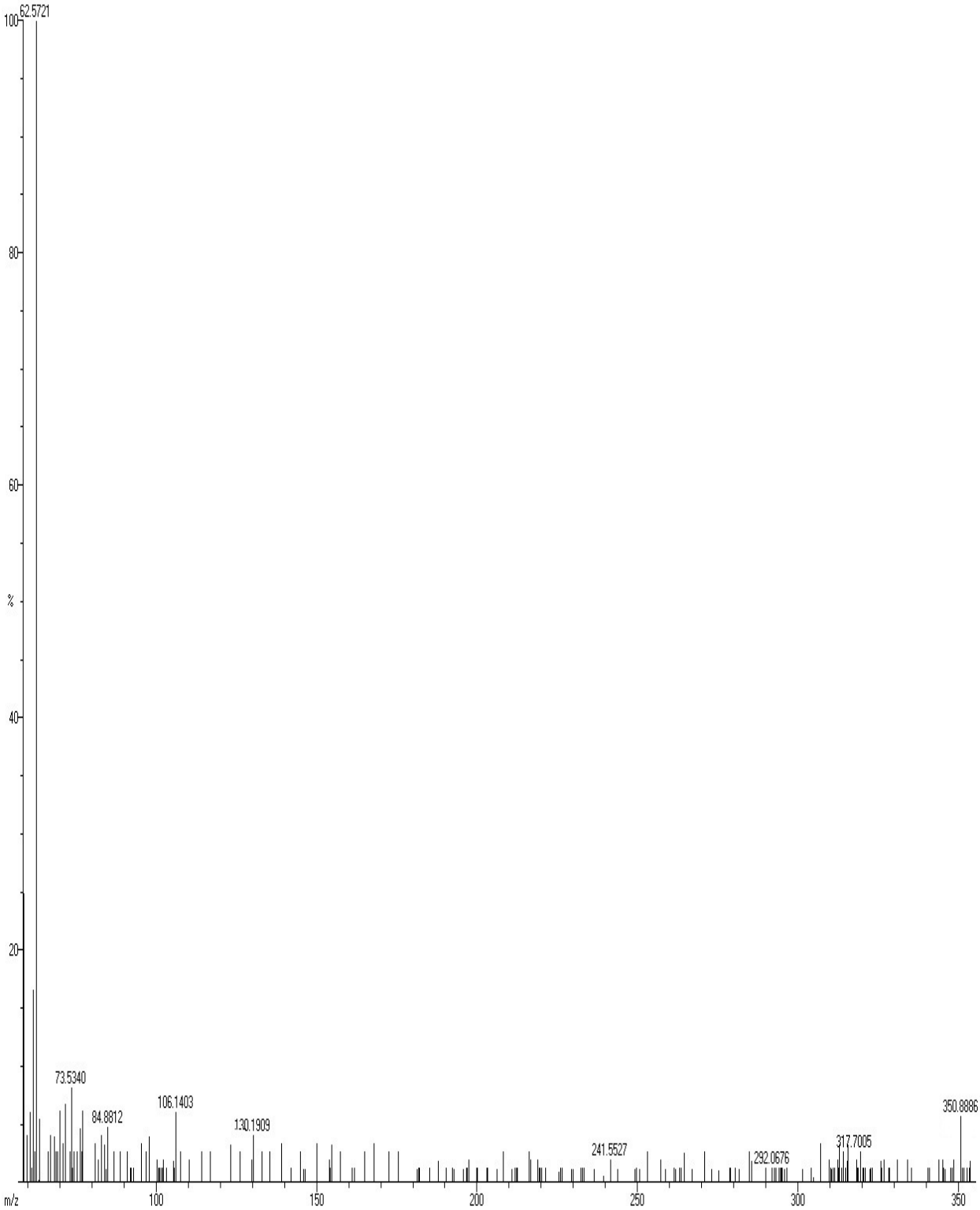


Fig. 25

5.4.Pharmacological evaluation:

Table 10:

5.4.1. Acute toxicity studies:

Behavioural and physical observations of mice following the administration of synthesized compounds for the dose limit of 300mg/kg body weight.

S.No	PARAMETER	0 min	1 hr	4hrs	8hrs	1 week	2 weeks
1	Straub's test						
2	Sedation						
3	Excitation						
4	Jumping						
5	Writhing						
6	Piloerection						
7	Stereotypy						
8	Scratching						
9	Grooming						
10.	Aggression						
11	Ptosis						
12	Exophthalmia						
13	Loss of righting reflex						
14	Loss of pineal reflex						
15	Loss of corneal reflex						
16	Salivation	Normal	Normal	Normal	Normal	Normal	Normal
17	Lacrimation	-	-	-	-	-	-
18	Skin and fur	Normal	Normal	Normal	Normal	Normal	Normal
19	Eyes	Normal	Normal	Normal	Normal	Normal	Normal
20	Tremors						
21	Diarrhoea						
22	Coma						

5.4.2. Cytotoxicity studies:

Compound 1:

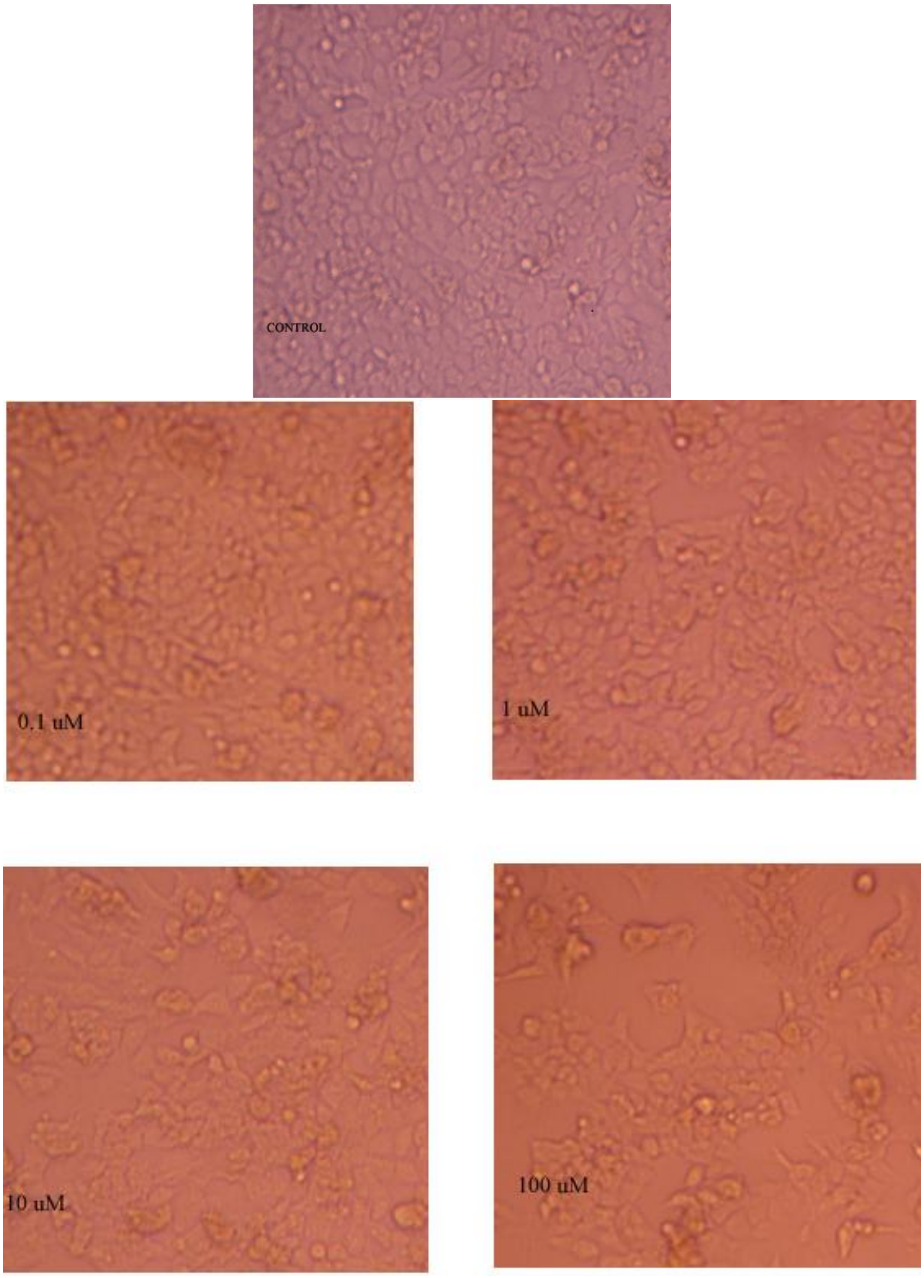


Fig.26

Table 11:

	Conc. µM/ml	**Absorbance**	**% inhibition**	**IC_{50} µM/ml**	**R^2**
C1	0.1	0.564333	1.684088	97.38	0.9911
	1	0.526	8.362369		
	10	0.473333	17.53775		
	100	0.281	51.0453		

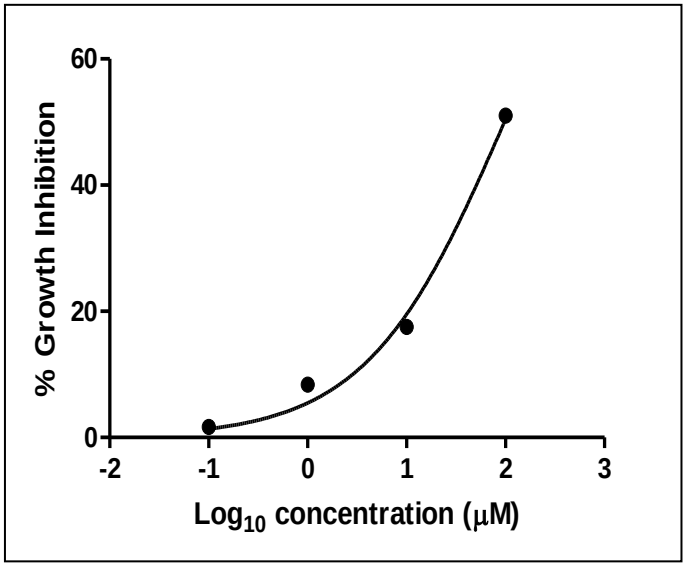


Fig. 27

Compound 2:

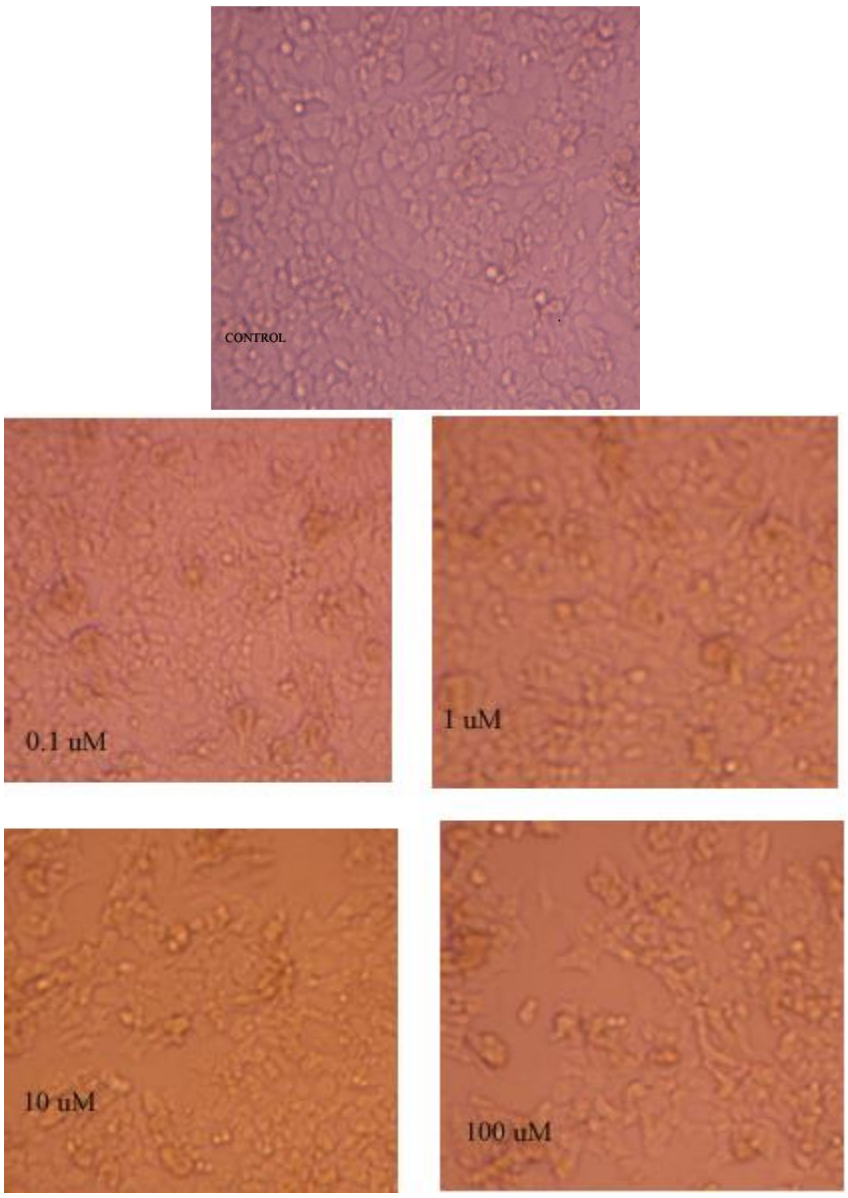


Fig. 28

Table 12:

	Conc. µM/ml	**Absorbance**	**% inhibition**	**IC_{50} µM/ml**	**R^2**
C2	0.1	0.571333	0.464576	113.2	0.9979
	1	0.553667	3.542393		
	10	0.51	11.14983		
	100	0.301	47.56098		

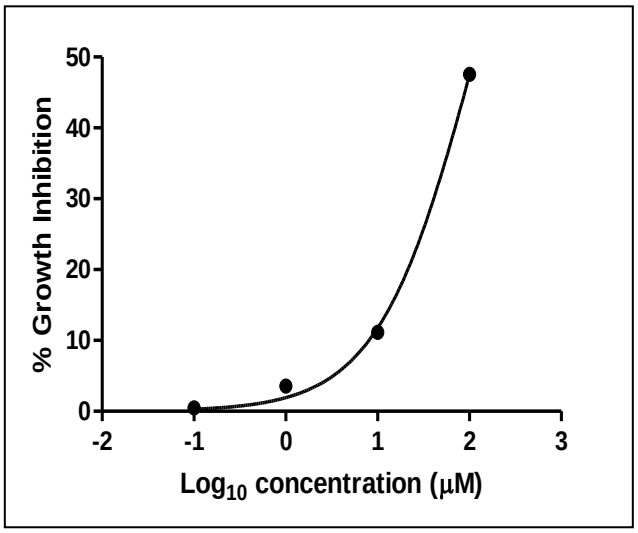


Fig.29

The cytotoxicity study was carried out for the synthesized compounds. All the compounds were screened for its cytotoxicity against human colorectal carcinoma cell line (HCT116) at different concentrations to determine the IC_{50} (50% growth inhibition) by MTT assay.

Results are tabulated in table 11& 12 and graphically represented in fig. 28&29.

The IC_{50} values of compound 1 and 2 were found to be 97.38 and 113.2 µM/ml and R^2 values were 0.9911 and 0.9979. These two compounds showed more significant effect on human colorectal carcinoma cell line (HCT 116) when compared to other compounds.

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

- Candidate molecules were docked for anticancer activity against the modeled protein target mTOR using drug design software (Maestro 9.1).
- Twenty five scaffolds were screened with high docking score against mTOR inhibitor. These compounds also passed Lipinski's rule.
- The scaffold containing quinoline nucleus was selected on the basis of synthetic feasibility.
- All the synthesized compounds were characterized by UV spectroscopy, IR spectroscopy, NMR spectroscopy and MASS spectrometry and reported as pure.
- All the synthesized compounds were subjected to acute toxicity studies to fix the LD_{50}. The LD_{50} value of the title compounds (C1-C5) was expected to be in category 4 i.e.

  > 300-2000mg/kg body weight.
- All the synthesized compounds were subjected to invitro experiment to determine anticancer activity using MTT assay procedure against mTOR inhibitor and found to be effective.
- Future studies:
  - ✓ Organ toxicity studies
  - ✓ Invivo anticancer studies

  The above studies have to be done to reveal that the synthesized compounds (C1, C2, C3, C4 and C5) have low side effect profile.

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7. BIBLIOGRAPHY

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